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Rhizome extracts of *Acorus odoratus*: Antifungal, anti-yeast, anti-oxidant and HPLC quantification

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Abstract

Different extracts from the rhizomes of *Acorus odoratus* were assessed for the presence of antifungal, anti-yeast and anti-oxidant potential. Mild antifungal and robust anti-yeast potential were revealed by the tested samples. A gigantic anti-oxidant activity, however, was noted for all extracts at each concentration used. The data further revealed that *n*-butanol and ethyl acetate fractions measured fair antifungal (41.5% inhibition against *Rhizopus oryzae* and 36.2% inhibition against *Acremonium alternatum* at 2000 µg/well respectively), and highest anti-yeast (70.3 and 66.8% activities respectively at 2000 µg/disc) and anti-oxidant (95.8 and 97.9% activities respectively at 250 ppm) potential while samples extracted with water were least potent. Furthermore, both *n*-butanol and ethyl acetate were found as the best extraction solvents for antifungal, anti-yeast and anti-oxidant compounds (particularly gallic acid) from the rhizomes of *A. odoratus* in HPLC analysis.

Introduction

The use of plants for therapeutic purposes is in vogue from time immemorial (Abraham et al., 1998). No less than 70-80% of the global population employ the use of herbal medication to cure their basic ailments (Srivastava et al., 1995).

A representative of the family Araceae, *Acorus odoratus* exists as a persistent herb alongside riverbeds and is also found in soggy wet areas. The potential of this plant as an antifungal (Lee et al., 2004; Lee, 2007), antibacterial (McGraw et al., 2002; Phongpaichit et al., 2005), allopathic (Nawamaki and Kuroyanagi, 1996), anticellular and immunosuppressive (Mehrotra and Mishra, 2003) agent has been known for quite some time. Conversely, the magnitude of work centered at uncovering its true potential as an antifungal, anti-yeast and anti-oxidant agent does not stand at par with the gigantic biological potency of its rhizomes. Furthermore, this study employed a novel approach wherein the rhizomes were extracted with different solvents in order of their ascending polarity. The need for such an experimental layout, therefore, was quite evident in illuminating the factual biological ability of *A. odoratus* rhizomes.

The accurateness, consistency and repeatability associated with HPLC (high performance liquid chromatography) contribute to its frequent use in polyphenol quantification from herbal extracts (Khan and Bakht, 2016). In this study, the different extracts were quantified for the presence of gallic acid using this technique for the aforementioned reasons.

Materials and Methods

Plant material

The rhizomes were collected from the different locations within the District Swat of Khyber Pakhtunkhwa Province, Pakistan. These rhizomes were thoroughly

washed to remove dirt particles, and were afterwards sliced and dried in shade. The completely dried rhizomes were then powdered in an electric grinder.

Crude extract preparation and its fractionation

The crude extract was prepared and fractionated according to the method described in our previous publication (Khan and Bakht, 2016).

Media used

Commercial products, procured from Oxoid Ltd., England, were used in the present study. For culturing of fungal and yeast strains, Potato Dextrose Agar (CM0139) was utilized, while the studied organisms were incubated and standardized in Potato Dextrose Broth (CM0001). Each media was prepared according to the protocol elaborated in Khan and Bakht (2016).

Fungal and yeast strains

The different fungi used in the study were *Trichoderma reesei* (ATCC # 26921), *Acremonium alternatum* (ATCC # 60645), *Aspergillus niger* (ATCC # 6275), *Rhizopus oryzae* (ATCC # 20344) and *Penicillium chrysogenum* (ATCC # 11709), while *Candida albicans* (ATCC # 10231) was the yeast used to test the biological potential of the extracts. All the tested organisms were collected from the Department of Plant Pathology, The University of Agriculture, Peshawar, Pakistan.

Antifungal, anti-yeast and anti-oxidant assays

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

HPLC quantification for gallic acid

Each extract was quantified for the presence of gallic acid using a liquid chromatograph (Shimadzu LC-6AD) fitted with dualistic pumps, a prominence diode array, PDA, detector (Shimadzu SPD-M20A), a communication bus module (Shimadzu CBM-20A) and C_{18} Diamonsil column (4.6 mm in diameter and 250 mm in length with particle size of 2.5 µM). Our aforementioned publication, Khan and Bakht (2016), can be referred to for a comprehensive overview of sample and standard preparation as well as HPLC method development.

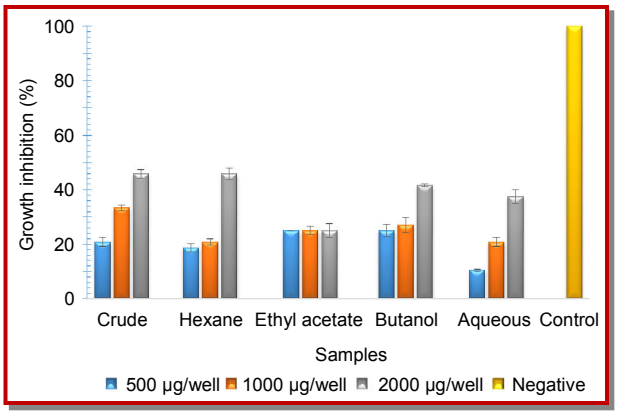

Figure 1: Antifungal activity of different extracts against *Rhizopus oryzae*

Statistical analysis

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

Results

Antifungal potential

The tested extracts were active only against *R. oryzae* and *A. alternatum*.

The antifungal potency of crude methanol extract and its fractions from the shade-dried rhizome of *A. odoratus* against *R. oryzae* is represented in Figure 1. All the tested extracts, at each concentration used, revealed inhibition activity against this organism. The crude methanolic extract, measuring 20.8, 33.3 and 45.8% inhibition at 500, 1000 and 2000 µg/well respectively, turned out to be the most effective among the tested extracts. The other extracts according to the descending order of activity were *n*-hexane (18.7, 20.8 and 45.8% inhibition at 500, 1000 and 2000 µg/well respectively), *n*-butanol (41.5% inhibition at 2000 µg/well) and water (37.5% inhibition at 2000 µg/well). Ethyl acetate fraction with an inhibition of 25.0% at 2000 µg/well, on the other hand, was the least effective. Likewise, this fraction was the sole among the tested with precisely an identical potency at each concentration used, i.e., 25.0% inhibition.

The different tested extracts were active, to varying degree, against *A. alternatum* (Figure 2). Nevertheless, the tested organism was found resistant to the 500 µg/well concentrations of both *n*-hexane and *n*-butanol fractions, and to the aqueous fraction at both 500 µg/well and 1000 µg/well. The least inhibitory activity was displayed by the water extracted sample measuring 16.3% inhibition at 2000 µg/well, while the highest activity was noted for hexane fraction which revealed 30.9 and 45.4% inhibition at 1000 and 2000 µg/well respectively. Methanol extract with an inhibition of 25.4 and 38.1% inhibition at 1000 and 2000 µg/well respectively followed hexane fraction in the descending order of activity. This in turn was followed by ethyl acetate and *n*-butanol fractions with an inhibitory activity of 36.2 and 27.2% respectively at 2000 µg/well.

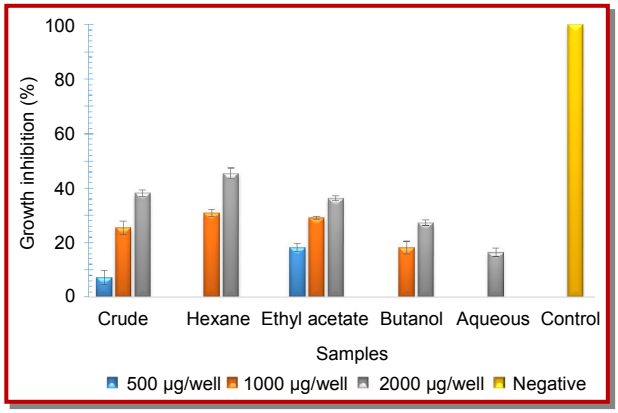

Figure 2: Antifungal activity of different extracts against *Acremonium alternatum*

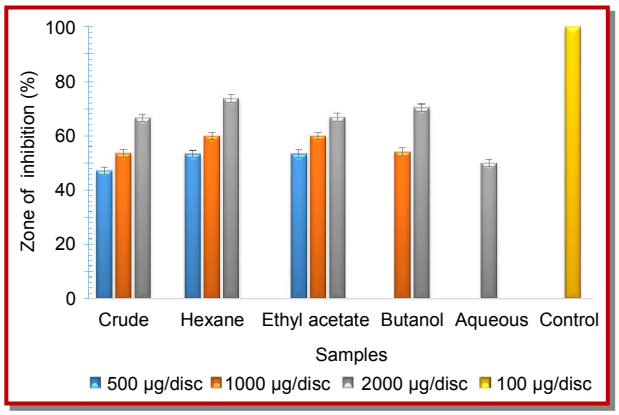

Figure 3: Anti-yeast activity of the different extracts against *Candida albicans*

Anti-yeast assay

Each concentration of all the tested extracts was active in inhibiting the growth of the yeast, *C. albicans*, excluding the 500 µg/disc concentration of *n*-butanol and the 500 and 1000 µg/disc concentrations of aqueous fraction. Furthermore, the least inhibitory activity of 50.0% at 2000 µg/disc was revealed by the water extract. *n*-Hexane fraction, on the other hand, with an activity of 53.3, 60.0 and 73.8% at 500, 1000 and 2000 µg/disc respectively was the most potent of the tested extracts against this organism. *n*-Butanol and ethyl acetate fractions (70.3% and 66.8% activity respectively at 2000 µg/disc), and methanolic extract with 66.6% activity at 2000 µg/disc followed suit (Figure 3).

Anti-oxidant Activity

All the seven concentrations of each tested extract were able to scavenge DPPH free radicals (Figure 4). Water extract with an activity of 72.5%, 72.7%, 73.5%, 75.3%, 77.8%, 78.7% and 82.9% at 5, 10, 25, 50, 100, 125 and 250 ppm respectively was the least potent among the tested extracts in this regard. Ethyl acetate fraction, conversely, measured an activity of 78.6%, 81.3%, 86.5%, 91.7%, 96.4%, 97.5% and 97.9% at 5, 10, 25, 50, 100, 125 and 250 ppm respectively and turned out to be the extract with the highest radical scavenging ability. Other extracts in order of descending potency were butanol fraction, methanolic extract and hexane fraction measuring 95.8%, 91.8% and 87.6% activity respectively at 250 ppm.

HPLC Analysis

The peak areas of the different gallic acid dilutions with respect to retention-time (RT) were plotted against their respective concentrations to obtain the regression equation and standard curve. The ppm concentration of gallic acid in each extract was calculated through the ensuing equation:

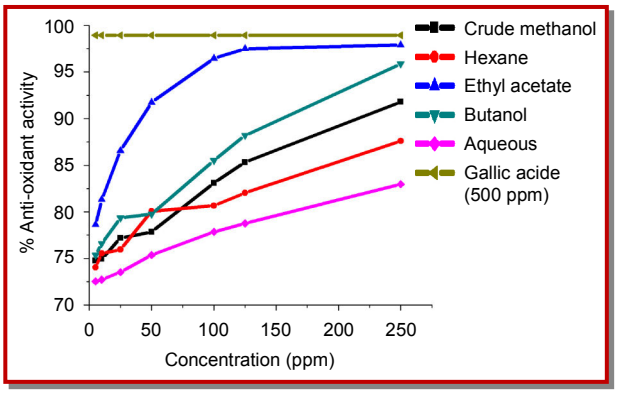

Figure 4: Anti-oxidant potential of the different extracts in DPPH radical scavenging assay

$y = 46598\ x + 62967$; $R^2 = 0.996$

In the above equation, the sample peak area is denoted by 'y' and gallic acid concentration in that individual sample is represented by 'x'

Furthermore, the amount of gallic acid (mg) in each gram of the dry extract for all the samples was calculated from the ppm concentrations obtained through the above equation. The mean value for gallic acid retention time with Standard deviation was 15.3 ± 0.2 min. Additionally, the identification of gallic acid peak in each sample was indicated by retention time and established by a comparison between the UV spectra of both.

A total gallic acid content of 38.4 mg/g dry extract was found in *n*-butanol fraction from shade-dried rhizome which was the highest among the tested samples (Figure 5). It was shadowed by ethyl acetate fraction having gallic acid in a concentration of 11.3 mg/g dry extract. The lowest concentration of gallic acid (7.2 mg/g dry extract) was calculated for water extracted sample.

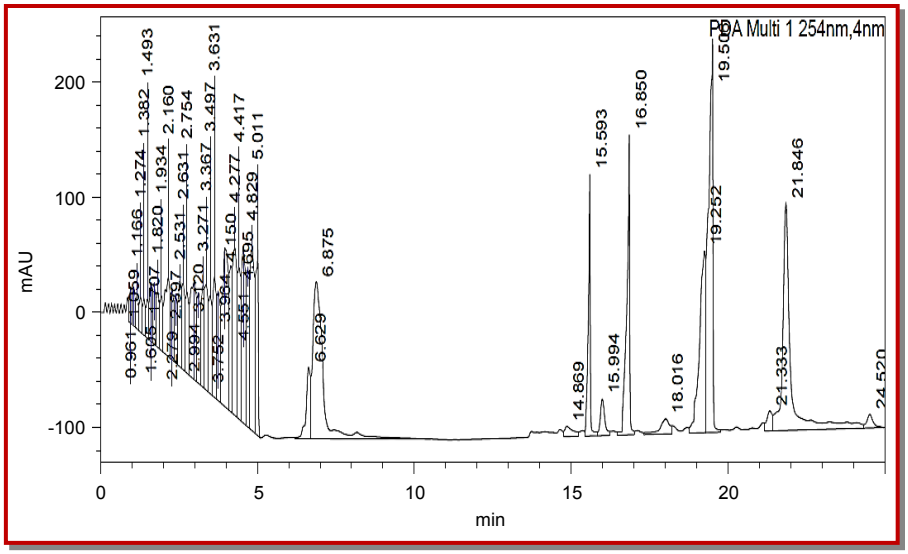

Figure 5: HPLC chromatogram of butanol extracted sample from shade-dried rhizome

Discussion

The methanolic extract and hexane fraction, revealing an identical activity at the highest concentration employed, were more able to control its growth than any other tested extract. Butanol, water and ethyl acetate fractions followed suit.

The human contagions caused by *A. alternatum* are habitually associated with immunocompromised individuals. The utmost recurrent contagion instigated by this organism is Mycetoma, which generally advances succeeding trauma. Some other infections associated with this organism are eye-infections, lung and gastro-intestinal tract colonization, and various other maladies *viz.*, osteomyelitis, sinusitis, peritonitis and arthritis (Fincher et al., 1991). All extracts inhibited the growth of this organism at each concentration employed, except hexane and butanol fractions which were inactive at the lowest concentration tested, and aqueous fraction which revealed some activity only at the maximum concentration used. Other extracts in plunging order of potency were hexane, methanol, ethyl acetate, butanol and aqueous fraction. These results can be best rationalized on the basis of polarity of the compounds extracted with diverse solvents (Manikandan et al., 2010).

There has been no earlier study, as per our knowledge, wherein either *R. oryzae* or *A. alternatum* has been used as test organism in uncovering the antifungal potency of extracts from any part of *A. odoratus*. The antifungal potential of extracts from different plant parts of *A. odoratus*, nevertheless, has been reported in various studies like Kumar et al. (2014), Singh et al. (2010), Devi and Ganjewala (2009), Phongpaichit et al. (2005) and Mungkornasawakul et al. (2002).

The yeast *Candida albicans* is a significant anthropological pathogen eliciting an array of ailments (Kim and Sudbery, 2011). Indiscreet fungiform contagions elicited by this yeast have amplified substantially (Cheng et al., 2005). As a cause of nosocomial circulation contagions in USA, they at present rank 4th (Jarvis, 1995) and represent the utmost typical one at a foremost sanatorium in Taiwan (Chen et al., 1997). Candidemia is frequently associated with prolonged stays in hospitals besides its concomitant fatality rate of thirty to forty percent (Leleu et al., 2002; Wey et al., 1988). Moreover, it is also linked with a paramount escalation in treatment expenses (Tortorano et al., 2004). Besides, majority of the prevailing prescriptions have detrimental characteristics, for example, "amphotericin B", because of its associated toxicity, harvests unsolicited side effects, "azoles" exhibit drug-drug interactions, and "fluconazole" and "5-flucytosine" lead to an onset of resistance (White et al., 1998). Developing an innocuous herbal treatment against this obstinate pathogen, henceforth, was of paramount significance.

Conveniently, the yeast was found susceptible to each extract from the rhizomes; some even revealed enormously encouraging outcomes. Hexane extracted fraction from shade-dried rhizome exhibited highest activity, trailed by butanol, ethyl acetate, methanol and aqueous fractions in a linear fashion. Comparable results can be found in the works described by Somnuk et al. (2014), Singh et al. (2011), Devi and Ganjewala (2009) and Phongpaichit et al. (2005). The findings of this work, on the other hand, are inconsistent with the results reported by Kumar et al. (2014) who testified that samples extracted from rhizomes of *A. odoratus* were more active against *Aspergillus niger* than against *C. albicans*. The extracts tested here, conversely, failed entirely in inhibiting the growth of *A. niger* and exhibited gigantic potency against *C. albicans*.

The assay wherein the potential of the extracts in

scavenging DPPH free radicals was assessed revealed extraordinarily auspicious results. Devi and Ganjewala (2011) also testified such high anti-oxidant activities for leaf and rhizome crude methanolic extracts *A. odoratus*. A mild potency in this regard of the essential oil (Shukla et al., 2012), and of root and rhizome ethanol and hydro-alcohol extracted samples (Elayaraja et al., 2010) has been previously reported. Tremendously lower anti-oxidant potency of rhizome water and methanol extracted samples, however, was stated by Manju et al. (2013). The employment of an entirely different extraction procedure than the one utilized here might be a factor contributing to such disappointing results.

The anti-oxidant potency of the extracts is also affected to a great deal by the diverse chemical features and polarities of the extracting solvents. The routinely employed solvents for this purpose include ethyl acetate, acetone, propanol and methanol (Alothman et al., 2009; Mahattanatawee et al., 2006). The anti-oxidants solubility in a particular solvent is an ultimate determinant of the potency of the extracts and hence, trying to develop a universal solvent with the ability to dissolve all anti-oxidants can be a tedious job. Maiden screening, therefore, is of supreme significance in the documentation of an appropriate solvent with the ability to convey peak scavenging potency to the extracts.

In the present study, methanol, hexane, ethyl acetate, butanol and water were used as solvents in assessing the scavenging potency of *A. odoratus*. Ethyl acetate fraction emerged as the best among the tested extracts in scavenging free radicals and it was narrowly shadowed by butanol fraction in this regard. Other extracts in descending order of effectivity were methanolic extract, and hexane and water fractions. Subathraa and Poonguzhali (2012), although employed an altered extraction procedure to that utilized here, reported virtually parallel results with *A. odoratus* rhizome water extracted sample. Ethyl acetate and butanol, thus can safely be regarded as the utmost appropriate solvents for anti-oxidants extraction from *A. odoratus* rhizomes.

Gallic acid is apparently a compelling antibacterial (Ravn et al., 1989), anti-inflammatory (Kroes et al., 1992) and anti-oxidant (Gramza et al., 2005; Karamać et al., 2005; Rice-Evans et al., 1996; Brand-Williams et al., 1995) mediator. Phenols are described as supportive in curing malignancy, cardiovascular and neuro-degenerative disorders, and likewise in demand by the cosmetic industry as antiaging merchandises (Gupta et al., 2012). These compounds connote the utmost pondered phytochemicals which have been comprehensively considered as prototypical arrangements in varied exploration zones (Boudet, 2007).

The rhizome sample extracted with butanol showed the highest quantity of gallic acid among the tested extracts. Ethyl acetate and water fractions followed suit in a sequential manner. Hence, butanol and ethyl acetate can be fairly advocated as the solvents of choice for phenolics, specifically gallic acid, retrieval from *A. odoratus* rhizomes. This particular polyphenol, however, cannot be efficiently retrieved in an aqueous medium based on the results obtained here. Gallic acid retrieval from various plant parts has also been reported to be satisfactorily achieved in organic solvents by many researchers like Sharma and Singla (2013), Gupta et al. (2012), Condrat et al. (2011), Deshmukh and Prabhu (2011), Singh et al. (2011), Singh et al. (2010) and Karamać et al. (2005). Additionally, the quantification data obtained through HPLC provides strong basis for the more robust biological activities of organic solvents extracted samples in comparison to the aqueous fraction as indicated by the different assays employed in this study.

Conclusion

Organic solvents were found to be the solvents of choice for the extraction of phenolics, particularly gallic acid, from the rhizome of *A. odoratus*. Water, conversely, failed to achieve satisfactory results in this context. Furthermore, the results also pointed towards the potential of gallic acid as an antifungal, anti-yeast and anti-oxidant agent.

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Conflict of Interest

There are no conflicts of interest to be declared.

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