Int J Pharm Bio Sci 2014 April ; 5 (2) : (B) 475 - 483

Research Article **Phytochemistry**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

EXTRACTION AND ANTIFUNGAL ACTIVITY OF TANNIN FROM TAMARIND HUSK

R.P.RAJADURAI JESUDOSS*, N.VASANTHI AND P.GAYATHRI

Department of Biotechnology, P.S.R. Engineering College, Sivakasi, Tamil Nadu, India.

ABSTRACT

Fungi are potent threat for food and tissue culture sector. Easy adoption and resistance of spores made fungus challenging to control. Natural antifungal agents are needed to control fungi in vegetables, foods, etc., in order to maintain foods natural character. In this study, we investigated the effect of various solvent crude Tannin extract (E) on the important food contaminating fungal agents like *Aspergillus niger, Aspergillus flavus, Aspergillus nidulans* and *Aspergillus fumigatus*. Tannin extracted from Tamarind husk using various solvents such as Cold water, Hot water, Acetone and Methanol (w/v) is used in this investigation. Antifungal activity was varied based on the solvent extract used, it was maximum in methanolic extract when compare to other modes of extraction. The crude methanol extract was purified by using column chromatography and the collected fractions were tested by ferric chloride test for the presence of tannin. We screened a maximum number of fractions and concluded that fraction 8^{th}, 9^{th} and 10^{th} showed significant result were subjected for antifungal activity.

KEY WORDS: Aspergillus species, Tannin, Column chromatography, Antifungal activity.

R.P.RAJADURAI JESUDOSS
Department of Biotechnology, P.S.R. Engineering College, Sivakasi, Tamil Nadu, India.

***Corresponding author**

INTRODUCTION

Fungi present ubiquitously in air and soil which are potent contaminants of foods. Fungal contamination is a major and frequent problem faced by food industry and tissue culture (plant) research centers all over the world. Some of the potent contaminants of food are species of *Cladosporium, Fusarium, Pencillium, Aspergillus,* etc [1, 2]. Among these *Aspergillus* species are commonly found as dominating contaminants in all sectors. *Aspergillus nige*r is a common fungus present ubiquitous in soil. It causes black mold diseases in fruits, vegetables and is a frequent contaminant of food [3]. When the spores of *A.niger* are inhaled it causes serious lung disease called Aspergillosis and it is deadly in case of inhalation of large amount of spores [4]. Initially it appears as white and matures as colonies (conidiophores) with a flat yellow basal covered by brown to black spores. It is long, globular structure and measures up to 4-5µm in diameter [5]. *Aspergillus flavus* is another potent causative agent for Aspergillosis and superficial infection [6]. It is commonly present in air and is more virulent than *A.fumigatus* a prevalent species of *Aspergillus* species [7]. It produces most toxic and potent natural compounds called aflatoxins [8]. *A.flavus* is also an agent for Keratitis, Otitis pulmonary and systemic infections in immunocompromised patients [9].Initial appearance of the fungi is a white colored flat sheet, and it matures into grassy green colored dense fibrous colonies. The conidia measures up to 3.5-4.5µm in diameter [10]. *Aspergillus nidulans* a filamentous fungus is also act as common contaminant of food by producing a carcinogenic mycotoxin from the precursor aflatoxin which causes mammalian liver cancer [11].It also serves as a model organism for the study of other *Aspergillus* species, on its harmful effects. *Aspergillus fumigatus* is a major contaminant of foods like cheese, smoked meats, pepper, herbal tea, downy-skinned fruits, smooth-skinned fruits, freeze-dried soups and even in individual food wrappings. It thread fully leads to the major opportunistic infection invasive Aspergillosis, primary gastrointestinal colonization and systemic infection in animals [12]. For controlling these food contaminating fungi, a number of studies and researches have been performed on various artificial & natural antimicrobial agents. Natural antimicrobial agents extracted from plant sources prove to exhibit potent antibacterial & antifungal activity. In plants, Tannin is a bioactive secondary metabolite [13] with the molecular weight of about 500-2000 Da [14]. It was found to be an effective antimicrobial agent over wide range of infectious bacteria. It is also known as proanthocyanidins that binds and precipitates proteins [15]. It is yellow to brown in color, present on the surface wax or within the vacuoles within the cell and it gets activated when the cell wall is being disturbed. It effectively functions as a defense mechanism against plant pathogens and adverse environmental conditions [16]. The astringent taste of wine and unripe fruits, enchanting color of flowers and autumn leaves are due to the presence of Tannin [17]. Tannin has a wide variety of useful applications such as antioxidant [18], anti-microbial [19], anti-inflammatory [20], anti-apoptosis [21], anti-aging [22] and cardiovascular protective agent [23]. Commercially, Tannin is extracted from various plant sources and marine brown algae [24]. The Tannins used for various purposes are most commonly obtained from the wood and bark of certain deciduous trees [25]. This study contributes to the extraction of Tannin from tamarind husk, an outer covering of tamarind pod (fruit) which is considered as a waste material and evaluation of its antifungal character against selected infecting fungus of *Aspergillus* species.

MATERIALS AND METHODS

(i) Isolation of microorganism

Fungus has been isolated from infected Amla (*Phyllanthus emblica*) and PTC media contaminant (Sunglow Biotech, Coimbatore-41). The fungus was cultured on Sabouraud Dextrose Agar [26] (HiMedia Laboratories Pvt. Ltd.) with the addition of antibiotic Ampicillin by streaking technique. The culture has been subcultured to obtain a pure culture.

(ii) Tannin extraction

(a) Pretreatment of Tamarind Husk

The tamarind husk (*Tamarindus indica*) was collected from the area Kothandaramapuram (Tirunelveli-15). The tamarind husk was grinded and made into fine powder which was then dried under sunlight for 8 hours to remove moisture content. The dried powder was stored at room temperature.

(b) Extraction of Tannin

The Tannin was extracted from the Tamarind husk using four different solvents such as hot water [27], cold water, Acetone [28] and Methanol [29]. In cold water extraction, 1g of dried tamarind husk powder was treated with 100ml of cold water by means of mortar and pestle. The solution was filtered through Whattman No.1 filter paper and the filtrate (E1) was collected and stored under refrigeration condition. In case of hot water extract, the dried tamarind husk powder (8g) was mixed with 150ml of water and it has been heated for 3 hours. The crude extract (E2) was obtained by filtering the solution through Whattman No.1 filter paper and it was stored under refrigeration condition. In Acetone extraction process, 400mg of dried Tamarind husk powder was weighed and it was pretreated with 40ml of Ethanol with 1% acetic acid. The solution was mixed for 5mins and it was filtered. The supernatant was discarded and the residue was collected. To the residue, 20ml of 70% Acetone was added and kept for 2 hours in shaking condition. After the process has been completed, the solution was filtered through Whattman No.1 filter paper. Then the supernatant was collected and it was found to be the crude extract (E3). In the Methanol extraction procedure, 10g of dried Tamarind husk powder was weighed and pretreated with 30ml of Ethanol with 52.8mg Ascorbic acid. The solution has been stirred for 45mins and then it was filtered through Whattman No.1 filter paper. The supernatant was collected and named as initial Methanol extract (E4). Then the residue was collected and treated with 7.5ml of Methanol with 13.2mg Ascorbic acid for 45mins stirring. Then the solution was centrifuged and the supernatant was stored. Again the residue was collected and the above two steps were repeated for 3 times. After 4 times of extraction, the supernatant was combined and filtered through Whattman No.1 filter paper. To the extract, equal volume of 0.05M Acetate was added which yields cloudy orange solution. Then the Methanol was completely removed from the extract by using magnetic stirrer at 30ºC. To the solution, 15ml of Ethyl Acetate was added into the separating funnel. After sometimes, the solution exhibits into 3 layers in which the lower phase was stored and the upper and the intermediate phase were discarded. To the lower phase the above 2 steps were repeated for 2 times. Then the lower phase was obtained at the third trial which was collected and evaporated by magnetic stirrer at 30ºC. To that equal volume of 80% Ethanol was added and the extract was stored. This was found to be the final Methanol extract (E5).

(c) Confirmatory test for Tannin

To confirm the presence of Tannin, the Ferric chloride test was done. 1ml of sample was taken and 2 drops of 5% Ferric chloride anhydrous ($FeCl_3$-162.21g/mol, s.d.fiNE-CHEM LiMiTEd, Mumbai-400 025) was added to 1ml of sample. Formation of green precipitate (Fig 1), confirms the presence of Tannin [30].

(d) Partial purification of Tannin

The crude extracts obtained from Methanol extraction was partially purified by column chromatography. The Silicon dioxide (SiO_2) was taken as a stationary phase. Dry Silicon dioxide (SiO_2) was sieved by 0.355mm size mesh. Sieved fine particles are then treated with magnets to remove charged particles. Column with the height of 31cm and width of 1.3cm was used. The packing of the column was done with silicon dioxide and acetone, bed height was found to be 23cm. 70% Acetone was taken as a mobile phase. The flow rate was calculated to be 1.2ml/min. After activating the column with acetone, 2ml of crude extract was loaded into the column. Samples are collected at equal intervals for each 2ml elute, and the presence of Tannin was confirmed by Ferric chloride test. The partially purified sample (E6) was stored under refrigeration.

(iii) Antimicrobial activity of Tannin

The antimicrobial activity of Tannin evaluated for the four fungus which is obtained from the source of infected Amla (*Phyllanthus emblica*) and PTC media contaminant (Sunglow Biotech, Coimbatore-41). The fungus has been cultured in Sabouraud Dextrose Agar (HiMedia Laboratories Pvt. Ltd.) with the addition of antibiotic Ampicillin. The fungus was cultured by means of spread plate technique. Wells of diameter 10mm were made and 0.2ml of different crude extracts and partially purified sample were loaded in the wells and the plates have been incubated at 37°C. After incubation, the zone of inhibition was measured.

RESULTS

The crude Tannin was extracted from the dried Tamarind husk powder by solvents such as Cold water, Hot water, Acetone and Methanol. The confirmatory test was done for all the extracts and the appearance of green precipitate indicates the presence of Tannin in all extracts except in cold water extraction. The color intensity of green precipitate was rich in the Methanol extraction indicates effective extraction of Tannin. Partially purification of methanolic extract by column chromatography using Silicon dioxide (SiO_2) at stationary phase yields partially purified Tannin. About 30 fractions of 2ml were collected and tested for Tannin presence by $Fecl_3$ test. The green precipitate was obtained for 8^{th}, 9^{th}, 10^{th} fractions which confirm the presence of Tannin (Fig 2). The purified sample was stored under refrigeration.

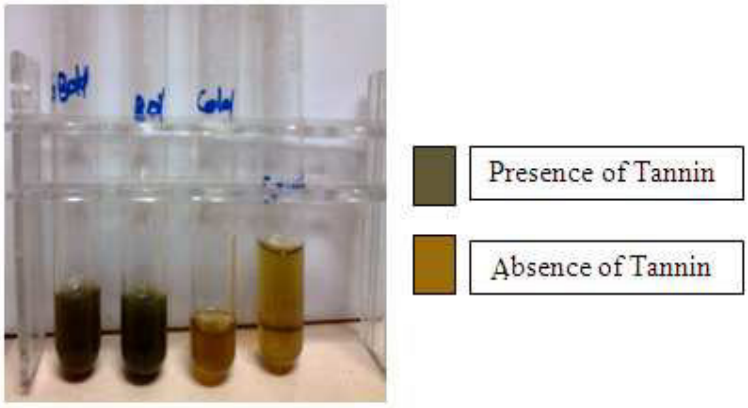


Figure 1
Confirmatory Test for Cold and Hot water extraction

Figure 2
Presence of Tannin in Column Fractions

Antifungal activity of crude Tannin extracted by different solvents was done against the fungus (*A.niger, A.flavus, A.nidulans, A.fumigatus*) isolated from the infected Amla (*Phyllanthus emblica*) and the PTC media contaminant. This inhibitory assay shows significant inhibition for Hot water, Methanol, and Acetone extractions. But Cold water extracts (E1) shows no significant inhibition. Hot water extracts (E2) shows significant inhibition to *A.nidulans* with a zone of 12mm in diameter and there is no significant inhibition to *A.niger, A.flavus* and *A.fumigatus.* In case of Acetone extracts (E3), which shows significant inhibition to *A.nidulans* and *A.fumigatus* with the zone of 18mm and 10mm in diameter respectively and there is no significant inhibitory effect to *A.niger* and *A.flavus.* Initial Methanol extracts (E4) shows inhibition to *A.flavus* with the zone of 14mm in diameter, *A.nidulans* with the zone of 24mm in diameter and *A.fumigatus* with the zone of 16mm in diameter except *A.niger* which shows no significant inhibitory effect. Final Methanol extracts (E5) shows significant inhibition to *A.nidulans* with the zone of 20mm in diameter and *A.fumigatus* with the zone of 12mm in diameter and there is no significant inhibition to *A.niger* and *A.flavus.* The partially purified Methanol extract (E6) shows significant inhibition to *A.niger* with the zone of 16mm in diameter (Fig 6), *A.flavus* with the zone of 24mm in diameter (Fig 5), *A.nidulans* with the zone of 36mm in diameter (Fig 3) and *A.fumigatus* with the zone of 22mm in diameter (Fig 4).

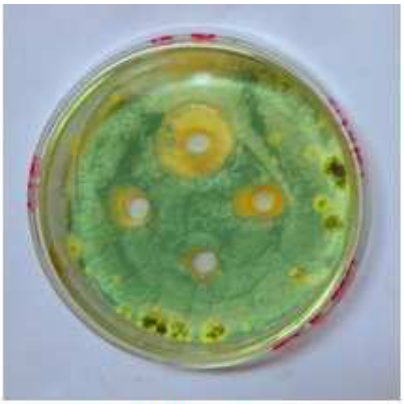


Figure 3
Inhibitory effect of Tannin for *A.nidulans*

Figure 4
Inhibitory effect of Tannin for *A.fumigatus*

Figure 5
Inhibitory effect of Tannin for *A.flavus*

Figure 6
Inhibitory effect of Tannin for *A.niger*

Table 1
Inhibition of fungal growth by various solvent extracts of Tannin

Fungus	Zone of inhibition				
	E1	E2	E3	E4	E5
A.niger	-	-	-	-	-
A.flavus	-	-	-	+	-
A.nidulans	-	+	+	+	+
A.fumigatus	-	-	+	+	+

Presence of Antifungal activity (+); Absence of Antifungal activity (-)

Graph 1
Effect of Tannin on fungal growth by various extracts

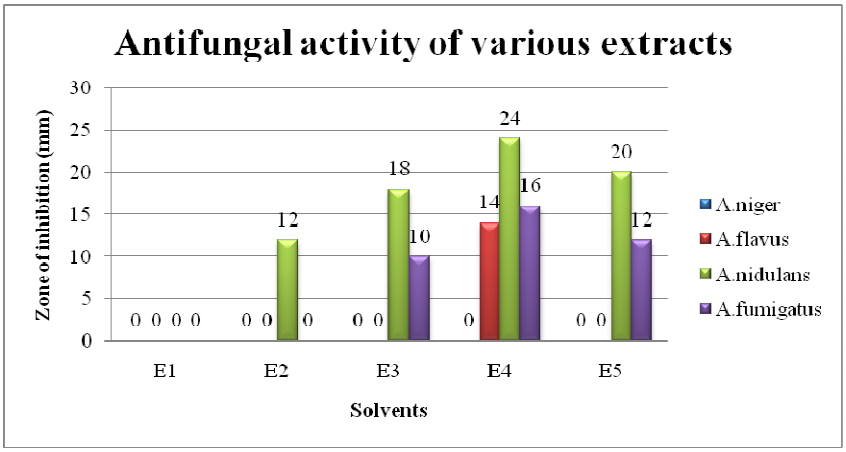
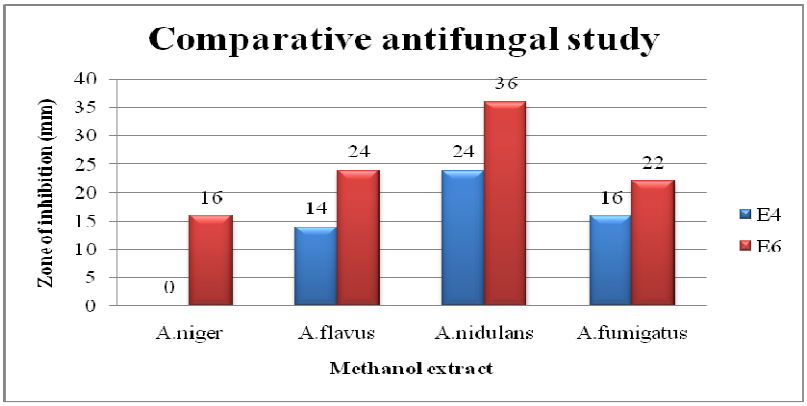


Table 2
Comparative study on antifungal activity for the crude (E4) and the partially purified (E6) Methanol extracts of Tannin.

Fungus	Zone of inhibition (Diameter in mm)	
	E4	E6
A.niger	0	16
A.flavus	14	24
A.nidulans	24	36
A.fumigatus	16	22

Graph 2
Comparative antifungal study of E4 and E6



DISCUSSION

Tannin has been previously extracted from various sources such as bark, leaves and stem of a plant and its antifungal activity was studied. This study deals with, extraction of Tannin from tamarind husk, which is a waste material only meant for ignition properties. The extraction of Tannin was efficient in Methanol extraction than in Acetone extraction. Previously, Tannin inhibitory study was done with different fungus but *Aspergillus spp* was rarely used. It has been seen that the extracts obtained by Cold water, Hot water, Acetone and Methanol shows inhibition to the growth of *A.niger, A.flavus, A.nidulans* and *A.fumigatus* at various extends. During our study, Tannin has shown effective inhibition on the fungus *A.nidulans.* Selime Mentes Colak *et al.* demonstrated the antimicrobial activity of Tannic acid in pickling process in which *A.niger* shows inhibition with the zone of 14mm diameter to the sample well containing 3% Tannic acid in 8mm well. To *A.flavus* the zone of inhibition was 12mm diameter to the sample well containing 3% Tannic acid in 8mm well. To *A.fumigatus* 11mm diameter zone of inhibition was formed in 8mm well containing 3% Tannic acid [31]. During this study, the antifungal activity of extracts containing Tannin on *A.niger* (Fig 8)shows a zone of 16mm diameter, *A.flavus* (Fig 7)with a zone of 24mm diameter, *A.fumigatus* (Fig 6)with a zone of 22mm and for *A.nidulans* (Fig 5)the zone of 36mm diameter were obtained. Thus methanolic extract of Tannin extracted from tamarind husk exhibits a significant inhibition over *A.nidulans* and *A.flavus*.

CONCLUSION

Tamarind husk seems to be a good source of Tannin. Methanolic extraction of Tamarind husk evidenced high extraction of Tannin. Extracted Tannin shows effective inhibitory effect over fungi *Aspergillus nidulans* and *Aspergillus flavus.* Thus Tannin acts as an effective antifungal agent against contaminating fungus.

ACKNOWLEDGEMENT

The authors highly acknowledge Head and all the Faculty members of Department of Biotechnology, P.S.R. Engineering College, Sivakasi for providing laboratory facilities and valuable guidance throughout this study.

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