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*Full Length Research Paper*

# Phytochemical screening and *in vitro* evaluation of anticandidal activity of *Dodonaea viscosa* (L.) Jaeq. (Sapindaceae)

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**The activity of fractions derived from hydroalcoholic extract of *Dodonaea viscosa* leaves against *Candida albicans* (Cl. I. 4043) was evaluated. The hydroalcoholic extract was sequentially fractionated to give n-hexane, dichloromethane, ethylacetate and n-butanol fractions that were subjected to qualitative phytochemical analyses. Disk diffusion assay was used in preliminary anticandidal screening with clotrimazole and chloroform serving as positive and negative controls, respectively. Optimized solvent systems were used for thin layer chromatography (TLC) that was followed by contact bioautography to evaluate the bioactivities of the fractions. Using broth microdilution technique, minimum inhibitory concentrations (MIC) of the individual fractions were established. With the exception of aqueous fraction all the fractions exhibited anticandidal activities (zone of inhibition 10 mm) in preliminary screening against test yeast. However, n-hexane fraction showed two inhibition zones at $R_f$ = 0.14 and 0.60 in contact bioautography, which indicates location of inhibitory compounds. The MIC of 62.5 µg/ml also supports the presence of anticandidal moieties in n-hexane fraction. Flavonoids, terpenoids, tannins and steroids were the main metabolites indicated in phytochemical screenings.**

**Key words:** *Dodonaea viscosa*, Sapindaceae, anticandidal activity, contact bioautography.

## INTRODUCTION

*Candida* species are part of normal microbial flora, yet they are endogenous opportunists. They are involved in the cutaneous and mucosal candidasis, systemic candidasis and chronic mucocutaneous candidasis. Most notable species is *Candida albicans* that is commonly seen in cases of oral thrush and vulvovaginitis (Forbes et al., 2007). Due to its pathogenicity and resistance issues, many studies focus on the ways and means of its prevention and cure. Angusticornin B and bartericin A, isolated from the twigs of *Dorstenia angusticornis* Engl. were found to be inhibitory to three *Candida* spp. thus indicating anticandidal potential of the flavonoids (Kuete et al., 2007). From liverwort *Asterella angusta*, ten dibenzofuran bis(bibenzyls)s including four new ones were separated and tested for antifungal activity against *C. albicans* using bioautography technique as well as broth microdilution method. The MICs were ranged from

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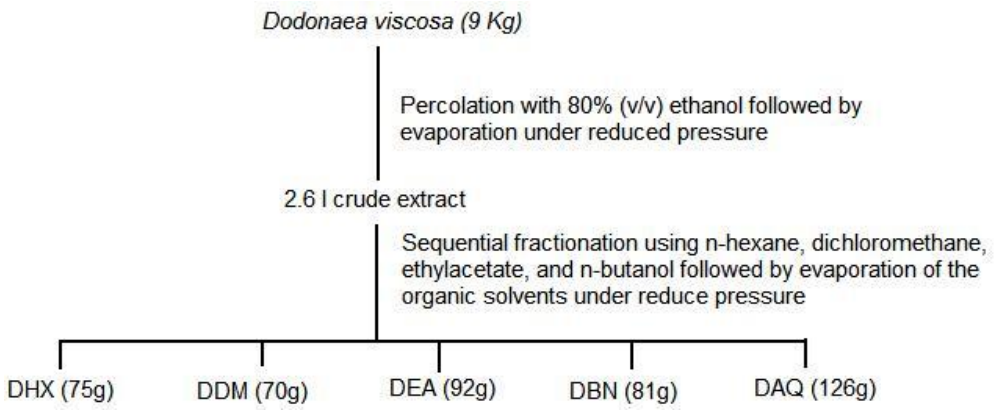


**Figure 1.** Extraction and fractionation of *D. viscosa*: (DHX = *D. viscosa* n-hexane fraction; DDM = *D. viscosa* dichloromethane fraction; DEA = *D. viscosa* ethyl acetate fraction; DBN = *D. viscosa* n-butanol fraction; DAQ = *D. viscosa* aqueous fraction).

16 to 512 µg/ml exhibiting a moderate antifungal potential (Qu et al., 2007). Significant activities of *Dodonaea viscosa* crude extract have been reported against gram-positive, gram-negative organisms as well as a *C. albican* strain (Rojas et al., 1992). Anticandidal activity in crude acetone-based extract of the leaves of *D. viscosa* has been reported when forty clinical isolates of *C. albicans* including twenty each from HIV- positive and HIV-negative patients and a separate control strain, with MIC range of 6.25 to 25 µg/ml (Patel and Coogan, 2008). Extract from the leaves was also found to inhibit the adherence of *C. albicans* to oral epithelial cell (Patel et al., 2009), and since adherence is the preliminary step of any microbial invasion, therefore, it has the ability to cause impact upon the crucial colonization step of an infection process.

There are significant concerns related to resistance problem in *Candida* spp. particularly *C. albicans*, especially against azole group antifungals (Perlin, 2010). From the available scientific data, it is evident that *D. viscosa* contains such principles that have antimicrobial potentials, but the molecules responsible for such activities are not yet completely marked. Since the plant is found abundantly in Pakistan, therefore, finding such principles in it can be very beneficial. The active components can be considered for induction into therapeutics as medicines after their *in vitro* and *in vivo* pharmacological evaluations and toxicological studies. This study was aimed to develop hyphenated bioactivity guided isolation technique that is sensitive, as well as cost effective for spotting of principles bearing activity against *C. albicans* from this plant.

## MATERIALS AND METHODS

### Chemicals, media and equipment

The pre-coated glass TLC plates (Kieselgel 60, $F_{254}$, layer thickness 0.25 mm) and all of the organic solvents were obtained from Merck, Darmstadt, Germany, except dichloromethane that was acquired from LabScan, Dublin, Ireland. Sabourad dextrose agar (SDA) and sabourad dextrose broth (SDB) were of Oxoid, Hampshire, UK. Clotrimazole, triphenyl tetrazolium chloride (TTC) was from Sigma-Aldrich, Steinheim, Germany and 96-well micro-titer plates were purchased from Sterlin, Aberbargoed, UK.

### Plant

*D. viscosa* (L.) Jaeq. belonging to the family Sapindaceae was collected from Kohat (Latitude 33° 19' 59" and Longitude 71° 10' 0" Pakistan) in September 2007. A specimen was matched for identity with the reference voucher number 592, preserved in the Herbarium of Pakistan, and was confirmed by Associate Professor Rizwana A. Qureshi, plant taxonomist, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

### Preparation of extract

The shade dried leaves with 1 to 2 cm portion of stems weighing 9 kg of *D. viscosa* were processed according to scheme (Figure 1) to yield respective fractions.

### Preliminary phytochemical screening

Phytochemical screening was carried out according to the methods described by Khandelwal (2006) and Edeoga et al. (2005) for alkaloids, steroids, tannins, triterpenoids, flavonoids, cyanogenic and coumarin glycosides. Briefly, Mayer's, Dragendorff's and Wagner's reagents were used to test the presence of alkaloids. Liberman-Burchard and Salkowski reactions were carried to test the presence of steroids. Triterpenoids were identified using Noller's reagent. Flavonoids presence was confirmed by Shinoda and Wolform tests. The presence of tannins was determined by reaction with 5% ferric chloride and gelatin. Guignard reaction that is carried out using a filter paper strip soaked in 10% picric acid and 10% sodium carbonate, respectively, and kept in slit of a cork over, the test material contained in a conical flask, brick red or maroon color indicates cyanogenic glycoside. While coumarin glycosides were evaluated by rendering alcoholic extract to an alkaline pH that if followed by appearance of blue or green fluorescence indicates their presence.

**Table 1.** Thin layer chromatography systems for *D. viscosa* fractions.

| Fraction | Mobile phase(s) used for development of chromatograms |
|---|---|
| DHX | 1.5% (*v/v*) $CH_3OH$ in $CHCl_3$ |
| DDM | Develop first with 5% (*v/v*) $CH_3OH$ in $CHCl_3$ then half develop with 30% (*v/v*) $CH_3OH$ in $CHCl_3$ |
| DEA | *n*-Butanol: Acetic acid: Water (12:3:5) |
| DBN | *n*-Butanol: Acetic acid: Water (12:3:5) |

**Table 2.**The susceptibility (expressed in mm mean ± SEM) of *C. albicans* (Cl. I. 4043) and minimum inhibitory concentrations (MIC) of *D. viscosa* fractions**.**

| Fraction | Zone of inhibition (mm) (n = 3) | MIC (µg/ml) (n = 3) | Controls | Zone of inhibition (mm) (n = 3) | MIC (µg/ml) (n = 3) |
|---|---|---|---|---|---|
| DHX | 13.23 ± 0.15 | 62.5 | *CLOT*† | 28.83 ± 0.60 | 0.39 |
| DDM | 9.83 ± 0.44 | >1000 | *$CHCl_3$*‡ | 0 | NT‡‡ |
| DEA | 12.17 ± 0.60 | 250 | *Ethanol*‡ | 0 | NT |
| DBN | 11.50 ± 0.29 | 500 | | | |
| DAQ | 0 | NT | | | |

‡$CHCl_3$ (Chloroform) and ethanol = negative controls; †CLOT (Clotrimazole) = positive control; NT‡‡ = Not tested.

**Preliminary anticandidal screening**

*C. albicans* (Cl. I. 4043) was maintained on SDA slants and refreshed fortnightly. The slants were stored at 4°C. Upon each subculture, the microorganism was gram stained for morphology and further characterized by simple germ tube test (Cheesbrough, 1987). A single isolated colony was aseptically transferred to SDB and incubated overnight at 37°C at 140 rpm in a shaker incubator. One hundred microliter of inoculum (ca. $5 \times 10^5$ CFU/ml) was evenly spread, with the help of a sterile glass spreader over the entire surface area of 15 cm diameter petri plates containing SDA. The seeded plates were allowed to dry under laminar flow. The disks containing the plant fractions, ethanol (negative control for fractions), 0.33% (*w/v*) clotrimazole in $CHCl_3$ that served as positive control, were prepared and applied using sterile forceps. The plates were placed in inverted position in refrigerator for a period of two hour in order to allow the materials to diffuse around the disks. The inhibition zones were measured after 48 h of incubation at 37°C in inverted position. The tests were run in triplicate.

**Thin layer chromatography and contact bioautography**

TLC chromatograms were developed using optimized solvent systems as shown in Table 1, and were examined under 254 and 365 nm ultra violet light wave lengths and metabolites were marked. Previously reported approach (Khurram et al., 2009) was utilized with slight modification to carry out contact bioautography experiments. The sterilized developed chromatograms were placed on the seeded plates (prepared as mentioned) aseptically and were left for a period of two hours, in order to allow the materials from them to diffuse on to the seeded plates. Clotrimazole disks (mentioned earlier) were used as positive control and the area submerged in the mobile phase in case of each fraction of TLC chromatogram served as negative control. Thereafter, TLC plates were removed from the surface with the help of sterile forceps and the plates were incubated in inverted position for 48 h at 37°C. The areas of inhibition were marked and relevant $R_f$ (retardation factor) values were recorded by comparing them with the TLC chromatograms. All the tests were run in triplicate.

**Susceptibility assay**

A broth microdilution method (Johann et al., 2007) using sterile flat-bottom 96-well micro-titer plates was applied to carry out MIC assays. Briefly, fractions were tested at eight concentrations that varied from 1000 to 7.8 µg/ml while positive control clotrimazole concentrations varied from 25 to 0.195 µg/ml. Inoculum was prepared as described earlier and was transferred in 100 µl volume in each of the test wells and controls. The plates were incubated at 37°C for 48 h. Activity in test wells was detected by adding 20 L of 1% (*w/v*) triphenyl tetrazolium chloride aqueous solution. MIC was defined as the lowest dilution of respective fraction that inhibited visible growth of test yeast, as indicated by the TTC color change from colorless to red after 3 h incubation at 37°C. Tests were run in triplicate.

**Statistical analysis**

Results were analyzed using two sample t-test at 95% level of significance ( = 0.05). Analysis was done using Statistical package Minitab v14 (Minitab Inc., Pennsylvania, USA).

## RESULTS

The 9 kg plant material of *D. viscosa* yielded 2.6 l of crude extract and fractionated (Figure 1) using the organic solvents in order of increasing polarity. The bioactivity of *D. viscosa* fractions against the yeast *C. albicans* showed good inhibitory potentials (zone of inhibition 10 mm). The results, given in Table 2, indicated that all the fractions were having activity against the test strain except for aqueous (DAQ) fraction. The

**Table 3.** $R_f$ values of metabolites and corresponding inhibition zones for *D. viscosa* fractions against *C. albicans* (Cl. I. 4043).

| Fraction | TLC (Metabolites observed at $R_f$) | Contact bioautography (Inhibitions observed at $R_f$) |
|---|---|---|
| *DHX* | 0.14± 0.01 , 0.42 ± 0.01, 0.54 ± 0.01, 0.60 ± 0.01, 0.66 ± 0.02, 0.72 ± 0.02, 0.80 ± 0.01, 0.84 ± 0.01, 0.87 ± 0.01, 0.90 ± 0.01, 0.94 ± 0.01 | 0.14, 0.60 |
| *DDM* | 0.35 ± 0.02, 0.42 ± 0.02, 0.45 ± 0.01, 0.49 ± 0.01, 0.57 ± 0.01, 0.61 ± 0.01, 0.81 ± 0.01 | --* |
| *DEA* | 0.04 ± 0.01, 0.07 ± 0.01, 0.31 ± 0.01, 0.61 ± 0.01, 0.86 ± 0.01, 0.91 ± 0.01 | -- |
| *DBN* | 0.04 ± 0.01, 0.07 ± 0.01, 0.27 ± 0.01, 0.57 ± 0.02, 0.76 ± 0.01, 0.91 ± 0.01 | -- |

*-- = No inhibition.

highest bioactivities were observed in the case of n-hexane (DHX) and ethyl acetate (DEA) fractions, with zone of inhibitions of 13.23 ± 0.15 mm and 12.17 ± 0.60 mm, respectively, while dichloromethane (DDM) fraction expressed lowest inhibitory effect of 9.83 ± 0.44 mm. It was observed that DHX was more effective than DDM (P = 0.009) and n-butanol (DBN) (P = 0.017) but had a statistically equal effect in comparison to DEA (P = 0.113). DDM had a statistically insignificant (P = 0.974) impact upon the control of the test yeast when compared with DEA. DAQ fraction and negative controls (pure chloroform and ethanol that were used to solubilize clotrimazole and test fractions, respectively), were found to pose no effect on the growth of the test yeast (P = 0.50). Clotrimazole, used as positive control, was effective against the test strain with zone of inhibitions of 28.83 ± 0.60 mm, and have had very significant impact in term of control of the test yeast, in comparison to all of the test fractions and negative controls (P < 0.05). The MIC data presented in Table 2, indicates DHX fraction to have highest inhibitory potential (MIC = 62.5 µg/ml). MIC of clotrimazole, used as positive control, shows that the yeast strain is sensitive to it.

On the basis of preliminary anticandidal screening of the fractions, contact bioautography was employed to test if a single compound or multiple compounds were responsible for the anticandidal effects of the separated fractions. Solvent systems used for the development of chromatograms (Table 1) and contact bioautography results for the respective fractions of plant are presented in Table 3. In the chromatograms eleven, $R_f$ points were observed for DHX fraction, seven for DDM, and six each for DEA and DBN fractions that did not only indicated the presence of separated metabolites, but also illustrated the diversity in these fractions. However, the results of contact bioautography revealed anticandidal activities only in DHX fraction at $R_f$ = 0.14 and 0.60. No inhibition was observed in case of any mobile phases used for separation of respective fractions. The contact bio-autography and MIC data of DHX suggests presence of some potential molecules having activity against the test microorganism.

## DISCUSSION

*Candida* spp. may cause severe nosocomial opportunistic infections. The resistance against various drugs used against *Candida* spp. is variable around the globe, yet it is increasing especially against azoles that serve as main drugs used for candidiasis (Wroblewska et al., 2002). *D. viscosa* has been used in folk medicine in various cultures of the world. The traditional antimicrobial uses include application as antibacterial, antiviral (Getie et al., 2003; Wagner et al., 1987), and anticandidal agents (Patel and Coogan, 2008). Phytochemical reports describe the presence of phenolics, terpenoids, and saponins (Rojas et al., 1992; Wagner et al., 1987; Sachdev and Kulshreshtha, 1986) in it.

In the present study, anticandidal activity was seen in all of the test fractions with the exception of aqueous fractions, with highest activity seen in the DHX fraction. This shows that fractionation resulted in the separation of the components to some extent that is obvious in the cases of complex mixtures of natural origin, especially obtained from the extraction of plant materials. But the true prospects of any of such plant indicating bioactivities can only be verified when the actual molecule responsible for activity is isolated and standardized for the said activity.

Since plants contain an enormous number of metabolites (Dixon, 2001) and, though separation can be

achieved by liquid, that is, liquid partitioning; the method adopted in this study at the start, yet it is impossible to obtain one single molecule from the fractions, mainly due to the close association of the molecules in the extracts and complex nature of plant metabolites, thereby, necessitating further extensive separation. Therefore, TLC was carried out, that resulted in further separation of metabolites. TLC is an easy and cost-efficient technique used in the separation of components of complex mixtures, commonly used for natural products. Suitable solvent systems can resolve effectively the compounds present in the test materials. The main benefits of this technique include low cost analysis, high-throughput screening of samples and minimal sample preparation (Wen et al., 2004). An additional benefit is that the chromatograms can be screened for antimicrobial activity. This separation technique can be hyphenated to microbiological assays like bioautography.

The limitations that can be associated with such methodologies include the masking effect of the secondary metabolites that may not get separated and therefore, eclipsing the effects of potential metabolites that may also not show their effects completely if they are present in extremely small concentrations. However, rapidity and simplicity of the methods can be utilized to get a first hand insight of antimicrobial activities of plants claimed to bear such potentials.

In the present study, the combination of TLC contact bioautography resulted in rapid identification of metabolites having anticandidal activity. This spotting of potential metabolites was further supported by the results obtained from MIC assays. Therefore, this approach can be adopted in the preliminary screenings of extracts, and fractions thereof to evaluate anticandidal potentials. Although, the fractions were active against test strain, as implicated in the disk diffusion assay, but when they were subjected to more extensive separation through TLC, the activity appeared to vanish. This indicates that it was probably due to the synergestic or potentiating potential of the secondary metabolites that was lost when they got separated during TLC. However, activity was present in only one fraction at two different $R_f$ points, which signifies their potential. Further work is underway to isolate the molecules indicating this activity.

## Conclusion

The experimental approaches not only justify the use of *D. viscosa* in traditional medicines, but also validate the techniques as rapid screening procedures for the identification of metabolites against such microorganisms. The methodology clearly defined the fraction having claimed antimicrobial activity.

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