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Research Article

# PHYSICO-PHYTOCHEMICAL ANALYSIS AND THEIR MINIMUM INHIBITORY CONCENTRATIONS OF VARIOUS EXTRACTS OF *DECALEPIS HAMILTONII* WIGHT & ARN AGAINST GASTROINTESTINAL DISORDER PATHOGENS

**SAMYDURAI P AND V THANGAPANDIAN***

**PG and Research Department of Botany Kongunadu Arts and Science College (Autonomous), Coimbatore-29, Tamil Nadu, India**
**Email: thangapandian2005@yahoo.com**

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**ABSTRACT**

The current study describes minimum inhibitory concentration and phytochemical constituents of petroleum ether, methanol and aqueous extracts of different parts of *Decalepis hamiltonii*. Phytochemical analysis of the crude extracts revealed the presence of tannins, Phlobatannins, saponins, flavonoids, steroids, terpenoids, cardiac glycosides and reducing sugar in this investigated plant. However phlobatannins were found to be absent in methanol extract of the root. Different solvent extracts were tested against four gastrointestinal pathogenic bacteria and a fungus, *Candida albicans* which causes skin disease. The bacteria include two gram positive, *Staphylococcus aureus*, *Bacillus subtilis* and two gram negative bacteria, *E. coli, Klebsiella pneumoniae*. 1ml of petroleum ether containing 0.098 and 0.049µg of extracts were recorded as MIC and MID respectively, which exhibited greater activity against tested pathogens.

**Keywords:** *Decalepis hamiltonii*, *Bacillus subtilis*, aqueous extract, cardiac glycosides

## INTRODUCTION

The *Decalepis hamiltonii* Wight & Arn belongs to family, Asclepiadaceae. It is an endemic and endangered medicinal plant[1]. This plant roots are seasonal and grow wild, which contains 2-hydroxy-4-methoxybenzaldehyde as a major compound. The fresh roots of *D. hamiltonii* are available during monsoon in Southern parts of India and are generally dried and preserved for various food and pharmaceutical applications[2,3]. Roots of *D. hamiltonii* have traditionally been used as demulcent, diaphoretic, diuretic and tonic. It is used for treatment of the loss of appetite, skin diseases, diarrhoea, nutritious disorders, blood purifier[4,5], epilepsy and central nervous system disorders[6]. Prevention of pathogenic and spoilage microorganisms in foods is usually achieved by using chemical preservatives. These chemical preservatives act as antimicrobial compounds, which inhibit the growth of undesirable microorganisms. Antimicrobial activity has also been reported[7]. *Escherichia coli* and *Staphylococcus aureus* are intestinal bacteria often implicated in several gastrointestinal disorders. Gastrointestinal diseases caused by *E. coli* are the most frequent causes of death in developing countries[8]. In 1998, the World Health Organization estimated that 80% of the people living in developing countries almost exclusively use traditional medicine. Most of the traditional medicines rely heavily on medicinal plants[9].

The main objective of this study is to determine the minimum inhibitory concentration of extracts taken from dried powder of this plant against four gastrointestinal disorder pathogenic bacteria and a fungus. Comprehensive literature survey indicated that systematic pharmacological work has not been done so far in this plant. Hence, this plant was selected to screen its antimicrobial activity.

## MATERIALS AND METHODS

### Plant material

Well grown and healthy fresh leaves, stem bark and roots of *Decalepis hamiltonii* were collected from the Eastern Ghats of Kolli hills, which is the inhabiting area of the Tribals.

### Phytochemical parameters

### Total ash

About 5g accurately weighed powder was incinerated in a silica dish put in a muffle furnace at the temperature not exceeding 450˚C until it become free from $CO_2$. It was then cooled and weighed.

### Water soluble ash

Ash was dissolved in distilled water and the insoluble parts were collected on an ash less filter paper and ignited at 450˚C to a constant weight. By subtracting the weight of insoluble part from that of the ash, the weight of soluble part of ash could be obtained. Percentage of water soluble ash was calculated with reference to the air dried ash (drug).

### Acid insoluble ash

Ash was boiled with 25ml dilute HCl (6N) for five minutes. The insoluble matter collected on as ash less filter paper, washed with hot water and ignited at a temperature not exceeding 450 ˚C to a constant weight.

### Preparation of extracts

### Solvent extracts

The leaf, stem bark and roots were washed in running tap water and air dried for 15 days. After that the plant parts were pulverized into fine powder using pestle and mortar. 50g of fine powder was packed with No.1 Whatman filter paper and placed in soxhlet apparatus along with solvent, petroleum ether followed by methanol. The residues were collected and dried at room temperature, 30˚C after which yield was weighed and then performed to activity.

### Aqueous extract

25g of leaf, stem bark and root powders were separately dissolved in 100ml hot distilled water containing conical flask that were kept on a rotary shaker for 12 hours under 80rpm and the residues were filtered using No. 1 Whatman filter paper. Then collected the residues were dried first on a hot water bath to remove wetness and then in an oven. After drying, the residues were weighed and scraped out and different aliquots were dissolved in 5ml sterile water and were stored at 4°C till used for further analysis.

### Primary phytochemical investigation

The extracts were screened for the presence of alkaloids, tannins, phlobatannins, saponins, flavonoids, steroids, terpenoids, cardiac glycosides and reducing sugar[10,11,12].

### Alkaloids

0.2g of extract was warmed with 2% $H_2SO_4$ for two minutes. It was filtered and few drops of Dragendroff's reagent were added. Formation of orange red precipitate indicates the presence of alkaloids.

**Tannins**

Small quantity of extract was mixed with water and then heated on a water bath. The mixture was filtered and ferric chloride was added to the filterate. Development of a dark green solution indicates the presence of tannins.

**Phlobatannins**

About 0.5g of each plant extract was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCL solution. Red precipitate formation shows the presence of phlobatannins.

**Saponins**

About 0.2g of the extract was shaken well with 5ml of distilled water and then heated to boil. Frothing evolution shows the presence of saponins.

**Flavonoids**

About 0.2g of each plant extract was dissolved in diluted NaOH and HCl. Yellow solution that turns into colourless indicates the presence of flavonoids.

**Steroids**

2ml of acetic anhydride was added to 0.5g of extract of each sample with 2ml $H_2SO_4$. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

**Terpenoids**

**Salkowski Test:** About 0.5g of each extract was dissolved in 2ml chloroform. Concentrated $H_2SO_4$ was carefully added to form a layer. A reddish brown colouration of the interface formation confirms the presence of terpenoids.

**Cardiac glycosides**

**Keller-killani Test:** About 0.5g of each extract was treated with 2ml of glacial acetic acid containing a drop of ferric chloride solution. This was underplayed with 1ml of concentrated tetraoxosulphate acid to give a brown ring formation at the interface.

**Glycosides**

Small amount of alcoholic extract of samples was dissolved in 1ml of water and then aqueous NaOH solution was added. Formation of yellow colour indicates the presence of glycosides.

**Reducing sugar**

The crude extracts were shaken well with 5ml of distilled water and filtered. The filtrate was boiled with drops of Fehling's solution A and B for 2 minutes, an orange red precipitate development indicates the presence of reducing sugar.

**Antibacterial activity**

To determine minimum inhibitory concentration (MIC) and minimum inhibitory dilution (MID) values, the microdilution assay as described by Eloff[13] was followed. Ampicillin was used as positive control and DMSO as negative control. Purified extracts were tested at an initial concentration of 100mg to 0.049µg/ml and the experiment was done in triplicate. The above mentioned bacterial organisms were used for activity, in MIC method. Briefly, 100µl of sterile distilled water was added to 96-wells of microtitre plates followed by the addition of 100µg to 0.049µg/ml purified extracts and serially diluted after which 100µl of the bacterial strains were added to all the wells of microtitre plates to give the final volume of 200µl. The prepared microtitre plates were sealed to avoid dryness and incubated overnight at 3℃ in 100% relative humidity. After overnight incubation an indicator of bacterial growth, 40µl of phenol red dissolved in water was then added to all the microtitre plate wells and incubated further for 30 minutes to 2h. Bacterial growth was indicated by the appearance of red colour and the wells that exhibited colourless or less in colour intensity indicated bacterial growth inhibition. The MIC values were then recorded as the lowest concentration at which a decrease in red colour is apparent compared to the next dilution after approximately 24h of incubation. The minimum inhibitory dilution (MID) (ml/g) indicating the volume to which the extract derived from 1g can be diluted and then still inhibit bacterial growth[14]. MID was also determined for each extract.

**Antifungal activity**

Antifungal activity against *Candida albicans* was performed using the micro-dilution assay described by Eloff[13], whose method was modified for fungi by Masoko[15]. Pure *Candida* culture was prepared in Yeast Malt (YM) broth that was incubated at room temperature for 24h and the absorbance was read at 530nm. From this stock, a 1:1000 dilution with sterile YM broth was prepared. Four hundred microlitres of each extract containing 50mg/ml was dissolved in 80% methanol. Thus two rows of serially diluted wells, 24 wells, were prepared out of 96-wells of microtitre plate by using sterile distilled water. One hundred microlitres of the dilute fungal culture were added to each well. Tetracycline was used as positive control, whiles the DMSO was used as negative control. The plates were covered with parafilm and incubated overnight at 37°C after which 40µl of growth indicator, phenol red was added to each well. If the wells remained clear, there was an inhibition. MIC values were recorded as the lowest concentrations that inhibited fungal growth after 48h. To determine whether the activity is fungistatic or fungicidal, 50µl of YM broth were added to the clear wells and they were further incubated for 24h. After which the last clear well was recorded as the minimum fungicidal concentration (MFC). The minimum inhibitory dilution (MID) (ml/g) and minimum fungicidal dilution (MFD) (ml/g) were prepared by dissolving 1g of derived extract in 1 ml of solvents (petroleum ether, methanol and water) that gave the stock solution. Various concentrations were prepared from 100 to 0.049µg/ml to find out MID and MFD, which can inhibit the growth or kill the fungal cells Eloff[14]. The assay was repeated thrice.

**Statistical Analysis**

Triplicates were maintained in all experiments and means were segregated using Duncan's Multiple Range Test (DMRT). Significant differences were recorded at 5% level ($P = 0.05$).

**RESULTS**

The investigated physical parameters from plant material were recorded in Table 1. The study of theses parameters would provide knowledge that will play important role in drug preparation.

**Table 1: Yield of total ash content and extracts obtained from various parts of *Decalepis hamiltonii*.**

| Plant parts | Solvent | Time of extraction | Colour of extract | Yield (%) | Physical parameters | Yield (%) |
|---|---|---|---|---|---|---|
| Leaves | PET | 24 h | Greenish | 12.10 | Total ash | 8.8% |
| | MeOH | 48 h | Dark Green | 56.55 | | |
| | Aqueous | 12 h | Dark green | 3.8 | | |
| Stem bark | PET | 24 h | Golden Yellow | 12.13 | Water soluble ash | 3.7% |
| | MeOH | 48 h | Dark Yellow | 58.99 | | |
| | Aqueous | 12 h | Dark Yellow | 6.6 | | |
| Root | PET | 24 h | Yellow | 15.5 | Acid insoluble ash | 2.56% |
| | MeOH | 48 h | Reddish | 72.25 | | |
| | Aqueous | 12 h | Reddish | 9.8 | | |

Yield (%) = (yield weight/sample weight) x 100

**Table 2: Preliminary phytochemical constituents of different solvent extracts of various parts of *Decalepis hamiltonii***

| Phytochemical constituents | Leaves | | | Stem bark | | | Root | | |
|---|---|---|---|---|---|---|---|---|---|
| | PET | MeOH | Aqueous | PET | MeOH | Aqueous | PET | MeOH | Aqueous |
| Tannins | + | + | - | + | + | + | + | + | + |
| Saponins | - | - | - | - | - | - | - | - | - |
| Alkaloids | + | - | - | + | - | + | - | - | - |
| Glycosides | + | + | + | + | + | + | + | + | + |
| Reducing sugar | - | - | - | - | - | + | - | - | + |
| Flavonoids | - | + | + | + | + | + | + | + | + |
| Phlobatannins | - | - | - | - | - | + | - | - | + |
| Steroids | + | - | - | + | - | - | - | + | + |
| Terpenoids | - | + | - | - | + | + | - | + | + |
| Phenol | + | + | + | + | + | + | + | + | + |
| Cardio glycosides | + | + | + | + | + | + | + | + | + |

-, Absent and +, Present

The results of phytochemical analysis of various solvent extracts of *D. hamiltonii* were recorded in Table 2. From this analysis, methanol extracts of stem bark and root were found to have more chemical constituents compared to other extracts.

**Antibacterial activity**

Table 3 revealed the MIC and MID values of different solvent extracts of various parts of *Decalepis hamiltonii*. Although wide concentration spectrum was used to measure MIC and MID values, 0.98 and 0.049µg of extracts in 1ml of solvent (petroleum ether or methanol or hot water) were found to be suitable for MIC and MID respectively. These concentrations could control tested two Gram-positive and negative bacteria, when root and stem bark extracts were used against them. 0.50µg of Ampicillin in 1ml of solvents were used as positive control. The aqueous extracts of root and stem bark exhibited lower MID values against the two Gram-positive and gram negative bacteria, except the same extract of leaf. In general, with some exception in root extracts, the MID values recorded both in methanol and aqueous extracts was generally higher than all the other extracts.

**Antifungal activity assay**

The antifungal activity of *D. hamiltonii* extracts against *C. albicans* is presented in Table 4. All the extracts showed inhibitory activity with MIC ranging from 1.65µg/ml. In general, all the extracts exhibited moderate fungicidal activity against *C. albicans*. The aqueous extracts had the lowest MID compared to other extracts. The aqueous extracts similarly had the lowest MFD compared to MeOH and PET extracts. Although, the inhibitory activities of the PET and MeOH root and stem bark extracts showed higher activities than that of the leaf. This observation probably suggests that the presence of the phytoconstituents in the root and stem bark extracts of *D. hamiltonii* have fungistatic activities, when compared to the leaf extract.

**Table 3: *In vitro* antibacterial sensitivity of different solvent extracts of various parts of *Decalepis hamiltonii***

| Plant parts | Extract | Minimum inhibitory concentration (MIC) (mg/ml) | | | | Minimum inhibitory dilution (MID) (ml/g) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *S. aureus* | *B.subtilis* | *E. coli* | *K. pneumoniae* | *S. aureus* | *B.subtilis* | *E. coli* | *K. pneumoniae* |
| Leaves | PET | 1.49 | 2.37 | 3.18 | 3.20 | 4.80 | 5.10 | 5.21 | 4.81 |
| | MeOH | 2.32 | 2.87 | 3.30 | 3.10 | 5.3 | 5.40 | 4.01 | 3.12 |
| | Aqueous | 2.20 | 1.21 | 2.10 | 3.12 | 6.1 | 5.38 | 5.35 | 5.41 |
| Stem bark | PET | 0.98 | 0.96 | 1.09 | 1.15 | 3.33 | 3.02 | 3.71 | 3.20 |
| | MeOH | 1.58 | 1.33 | 1.35 | 1.13 | 3.18 | 3.33 | 3.43 | 3.41 |
| | Aqueous | 2.13 | 2.90 | 2.41 | 3.23 | 2.0 | 2.10 | 2.02 | 2.60 |
| Root | PET | 0.76 | 0.78 | 0.88 | 0.91 | 1.40 | 1.66 | 1.35 | 0.62 |
| | MeOH | 0.98 | 0.90 | 0.79 | 0.89 | 1.1 | 1.13 | 1.70 | 1.12 |
| | Aqueous | 1.12 | 1.30 | 1.10 | 1.96 | 2.1 | 3.33 | 1.12 | 1.30 |
| Ampicillin (µg/ml) | | 0.188 | 0.170 | 0.211 | 0.230 | | | | |

Ampicillin - Positive control

**Table 4: *In vitro* antifungal sensitivity of different solvent extracts of various parts of *Decalepis hamiltonii* against *Candida albicans***

| Plant parts | Extract | Minimum inhibitory concentration (MIC) (mg/ml) | Minimum fungicidal concentration (MFC) (mg/ml) | Minimum inhibitory dilution (MID) (ml/g) | Minimum fungicidal dilution (MFD) (ml/g) |
|---|---|---|---|---|---|
| Leaves | PET | 4.66 | 3.21 | 8.65 | 4.20 |
| | MeOH | 2.70 | 6.10 | 7.10 | 8.32 |
| | Aqueous | 6.10 | 7.3 | 4.13 | 4.78 |
| Stem bark | PET | 1.66 | 2.80 | 2.45 | 1.80 |
| | MeOH | 2.11 | 3.92 | 3.55 | 2.51 |
| | Aqueous | 2.25 | 4.21 | 4.01 | 4.07 |
| Root | PET | 0.61 | 2.80 | 2.88 | 3.04 |
| | MeOH | 1.00 | 1.01 | 1.68 | 3.65 |
| | Aqueous | 1.15 | 1.16 | 1.09 | 2.06 |
| *Tetracycline (µg/ml) | | 0.044 | 0.158 | | |

* Tetracycline-Positive control

## DISCUSSION

Rhizome of *Decalepis hamiltonii* is largely used in South India for pickling along with curds or lime juice[16]. Phytochemical analyses of this plant have confirmed the occurrence of Terpenoids, Phenol, glycosides, flavonoids and saponins. Plants have been utilized as an important source of medicines, as they are the reservoirs of chemical agents with antimicrobial properties[17]. Earlier reports of the phytochemical analysis of the roots revealed that 2-hydroxy-4-methoxybenzaldehyde is the important and major component of the plant[18]. This plant derived 2-hydroxy-4-methoxy benzaldehyde phytochemical components are flavonoids, which may be responsible for their antimicrobial properties. Antimicrobial properties of *D. hamiltonii* have been reported by many workers, who involved only human pathogenic microorganisms[19,20,21,22]. The insecticidal property of this compound against important storage tank insects (*Sitophilus oryzae* L., *Rhizopertha dominica* F. and *Tribolium castaneum* Hbst.) has also been demonstrated[23,24]. Mohana[25] has reported antifungal activity of *D. hamiltonii* against phytopathogenic fungi. Similarly an essential oil and 2-hydroxy-4-methoxy benzaldehyde were also derived from *Periploca sepium* of Asclepiadaceae. These phytochemicals exhibited antimicrobial activities against all the test bacteria and *Candida albicans.* The tribes of central India used plants as traditional folk medicine for antibacterial activities[26]. MBC/MFC values observed range from 125µg/ml to 300µg/ml, MIC values from 80µg/ml to 300µg/ml, and $IC_{50}$ values from 63.29µg/ml to 167.30µg/ml[27].

## CONCLUSION

Though various parts of the plants were used for extraction, the extracts taken from the dried tuberous root and stem bark of *Decalepis hamiltonii* inhibited all the microbes involved in this study with minimum inhibition concentration ranging from 0.098µg and 0.049µg/ml. This study showed that crude methanol extracts of this plant could inhibit certain gastrointestinal disorder causing pathogenic bacteria, *Escherichia coli* and *Staphylococcus aureus*. Hence, the crude extract can be used as natural antibacterial compounds to prevent the growth of undesirable microorganisms; thereby gastrointestinal disorders can be controlled. Or a new formulation can be designed to develop a novel drug.

Hence, this study suggests that either the crude extract can alone be used as natural antibacterial compounds to prevent the growth of undesirable microorganisms; thereby gastrointestinal disorders can be controlled or a new formulation can be designed to develop a novel drug.

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