Antimicrobial, Phytochemical Screening And Identification Of Chemical Constituents Ofextracts Of Moullavaspicata(Dalzell) Nicolson

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Abstract-

Background:*Moullavaspicata(Dalzell) Nicolson is an endemic medicinal plant, it is robust, woody, shrub foundin Maharashtra.*

Aim: *Extracted Phytochemicals of this plant, to be detected and screened for their antimicrobial activities.*

Materials and Methods: *Three solvents were used for extraction of phytochemicals form root andstem by Soxhlet method. Antimicrobial assay and phytochemials were detected bydiffisuion method and biochemical test respectively. Chemical constituents wereanalyzed by GC-MS.*

Results*: Most of the phytochemicals were present in aqueous and hexane extract of stem and showed antifungal activity (Candida albicans,aspergillusniger)and antibacterial activity (S. aureus). Antimicrobial activities against thefungi and bacteriumshowed zone of inhibition of 10 mm for Candida albicans, 3mm for S. aureus. Extract showing both activity was analyzed by GC-MS analysis.*

Conclusion:*Promising antifungal activity (Candida albicans,Aspergillusniger) and antibacterial activity (S. aureus) suggests its use against these fungi and bacterium.*

Keywords- Antimicrobial activity, Moullavaspicata(Dalzell) Nicolson, GC-MS

I. INTRODUCTION

Compounds derived from plants are being widely used for their nutritional and chemotherapeutic values. (Zeng et.al., 2012, Gull et., al. 2012). Pharmacological dose of medicinal plants by traditional practitioners remains the mainstay of some health care system in the rural areas of developing countries (Hogg, 2002, Adenyemiet., al. 2012). 74% of 119 % plant-derived pharmaceutical remedies are used on the basis of their correlation with traditional uses as medicinal plants (WHO, 2000). Plants have been significantly used for medicinal purpose. 65% of people use medicinal plants for the needs of health care, in India (WHO, 2010). Medicinal plants including vegetables and spices contain various health-promoting and effective constitutes that include tannins, alkaloids, terpenes and secondary metabolites (Hogg, 2002, Manach, 2004, Arts and Hollman, 2005).

Moullavaspicata(Dalz) Nicols is an endemic and traditional plant, a montotypic genus of *Leguminosae*(Caesalpiniaceae) commonly called *Waghati*. It is robust, woody, climbing or scandent shrub growing abundantly in Maharashtra State, especially in hilly regions of Konkan belt (part of Western ghats). Branches are armed with numerous recurved prickles. Leaves are 8 to 30 cm long; rachis armed with prickles; pinnae 4-6 pairs, 5-12 cm long, flowers are nearly sessile reaching up to 60 cm long, pods 3-6 x 1-1.5 cm linear-oblong, torulose with thickened sutures. Chopra et. al., (1956) have discussed about the application of bark extract in the treatment of skin diseases. Bhatnagar et.al, (1961) reported the antibacterial activity of roots. Plant possesses antiseptic properties (Chopra et. al., 1958) and its effects on respiration and on cardiovascular (Dhar et. al., 1973). Medicinal plant materials are readily available in rural areas at relatively cheaper than modern medicine (Mann et al., 2008). Plant products still remain the principal source of pharmaceutical agents used in traditional medicine (Ibrahim, 1997; Ogundipe et al., 1998). The effects of plant extracts on bacteria have been studied by many researchers in different parts of the world (Reddy et al., 2001; Ateb&ErdoUrul, 2003). Natural products are source of metabolites like tannis, terpeniod, alkaloids, flavonoids, etc. which act against anti-infectious pathogens and are cheaper. (Taylor et al., 1996; Amani et al., 1998; Cowan, 1999; Dahanukar et al., 2000, Mahesh and Satish, 2008). Medicinal plants are used by 80% of the world population as the only available medicines

especially in developing countries (Hashim et al. 2010). Therefore the objective of this analysis was to understand the antimicrobial properties of various part of *Moullavaspicata* (Dalzell) Nicolson and analysis of chemical constituents which are responsible for biological activity.

II. MATERIALS AND METHODS

2.1. Plant material:

Root and stem of *Moullavaspicata* were collected from Satara district, Maharashtra, India and authenticated and confirmed by Dr. B. M Gaiker (Co-author) Head, Post Graduate Department of Botany, Ahmednagar College, Ahmednagar, Maharashtra, India and deposited in the herbarium of the College, (ACAH 295). The collected root and stem were washed and dried in hot air oven for 2 to 3 days to reduce the moisture contents.

2.2 Extraction:

Dried plant material (140 gm) was obtained. 37.5 gm of stem and root powder were used for extraction with petroleum ether, hexane and water using Soxhlet.

2.3 Phytochemical screening:

Chemical test were carried out on the petroleum ether, hexane and aqueous extracts using standard procedures to qualitatively analyse constituents present. The identification of the phytochemicals was done as described by Harborn and Harborn (1988), Brindha et al. (1977).

1. Test for Tannins: 200µl of 5% ferric chloride was addedin 100ul of extract. Greenish black colour indicated tannins.
2. Test for Saponins: 200µl of extract and 200µl of distilled water formation layer of foam indicated saponins.
3. Test for Flavonoids: 100µl of extract and 500µl of dilute ammonia were mixed with few drops of conc. sulphuric acid. Yellow colouration indicated flavonoids.
4. Test for Alkaloids:1ml of extract, 1ml of conc. sulphuric acid were mixed with few drops of Mayer's reagent. Green colour indicated alkaloids.
5. Test for anthocyanin and betacynine: 200µl of extract, lml of 2N sodium hydroxide were added and heated for 5 min at 100°C. Yellow colour indicated anthocyanin.
6. Test for Quinones: To 500µl of extract, l ml of conc. sulphuric acid was added. Red colour indicated quinones.
7. Test for Glycosides: 200µl of extract, l ml chloroform and 10% ammonia solution were mixed. Pink colour indicated glycosides.
8. Test for Terpenoids: To 200µlof extract, 400µlof chloroform conc. Sulphuric acid was added. Red brown colour at the interface indicated terpenoids.
9. Test for Phenols: 100µlof extract, 200 µl of distilled water was added and few drops of 10% ferric chloride.Green colour indicated phenols.
10. Test for Cardiac Glycosides: 100 µl of extract, 400 µl of glacial acetic acid and few drops of 5% ferric chloride were mixed. On adding 50µl. of sulphuric acid brown ring at the interface indicated the cardiac glycosides.
11. Test for Carbohydrates: To 200 µl of extract, 200µl of iodine was added purple colourindicatedcarbohydrates.
12. Test for Steroids: This test was done by using Salkowki's test.

2.4 Quantitative analysis of alkaloids, saponin and flavonoids

2.4.1. Quantitative determination of alkaloids using Harbone Method

1 gm of powdered sample of both root and stem were taken in separate 250 ml conical flask. To this was added 40 ml of 10 % acetic acid in ethanol, in each of the flask containing the powdered samples. Both the flasks were covered and allowed to stand for 4 hours. The mixture was filtered through filter paper. The filtrate was concentrated on water bath to one third volume. Ammonium hydroxide was added drop wise till precipitation was observed. The precipitates were allowed to settle and collected through filtration and were washed with dilute ammonium hydroxide. The weight of the precipitate was noted.

2.4.2. Quantitative determination of saponin by Obadoni and Ohuko method (2001)

5 gm of each plant sample were taken separately in 250 ml conical flask and were mixed with 25 ml of 20 % aqueous ethanol. These samples were heated in water bath for 4 hours at 55^0C with intermediate stirring. The mixture was filtered through filter paper and subjected the above process again, but this time with 50 ml of 20 % aqueous extract. The extract was reduced to 10 ml by heating in the water bath at 90^0C and transferred to a separator funnel along with 5 ml of diethyl ether which was shaken vigorously. The aqueous layer

was extracted and the ether layer was discarded, 15 ml of n-butanol was added to the aqueous extract. To this n-butanol and extract solution were added 5 % aqueous sodium chloride and centrifugation of the sample was carried out. The pellet was evaporated in water bath, and its dry weight was noted.

*2.4.3. Quantitative determination of flavonoids by Bohm and Kocipai-Abyazan method (1974):*1 gm of each plant sample was extracted with 10 ml of 80 % aqueous methanol, repeatedly. The solution was filtered each time. The filtrate was transferred to a petri dish and evaporated to dryness over a water bath and weighed.

2.5. Antimicrobial Assay:

2.5.1. Antifungal Activity: Three plates of each organism were spread to see its antifungal activity. The antifungal activity of the plant extracts were studied by monitoring the zone of clearance. Each experiment was repeated five times.

2.5.2 Antibacterial Activity: Three plates of each organism were spread to see its antibacterial activity. The antibacterial activity of the were studied on bacterial species *staphylococcus aureus.*

The microorganisms required for the antimicrobial assay were freshly sub cultured from previous pure culture and were maintained. The suitable medium for the suitable organism were prepared accordingly.

Fungal microorganisms were spread on PDA plates and bacterial microorganisms were spread on NA plates. Four wells were made in the medium, and were loaded with 25 µl the extracts and solvent as control and kept for diffusion. The zone of inhibition were then measured.

2.6. GC-MS analysis:

Hexane and petroleum extract of stem and root were analysed on a gas chromatography/mass spectrometer (GCMS-QP 2010; Shimadzu, Kyoto, Japan).The samples were loaded into a RTX-5 capillary column (thickness-0.25 um; length-30 m, diameter-0.25 mm). The temperature program was set as follows: the initial temperature of column was 100°C, holding for 1 minute, increasing to 250°C at 15°C/min, holding for 10 minutes; injection temperature: 250°C. The ion source of the mass spectrometer was set at 200°C. The scanning *m/z* range was 40–600 atomic mass units. A 1-µl aliquot of sample was injected and the injector was split less type. The carrier gas was helium with a flow rate of 1.0 mL/min. Detection was performed by mass detector and constituents were identified by computer search using digital libraries of mass spectral data (NIST, 1998), and comparison of their retention indices and authentic mass spectra (Adams,2007). The peaks were identified using the NIST database and retention index.

III. RESULT AND DISCUSSION

3.1. Extractive value of stem and Root extract

The extractive values were determined on the basis of percentage of dry weight residue after complete evaporation of solvent. Extractive value of solvent indicates that more phytochemicals were extracted in distilled water (2.221%) as compared to petroleum ether and hexane. However, slightly more phytochemicals were extracted in hexane (0.962%) as compared to petroleum ether. But in case of root extract, petroleum ether showed more extractive value (1.927%) as compared to other studied solvent as depicted in table 1.

Table 1
Extractive Value of phytochemical of *Moullavaspicata* (Dalzell) Nicolson in petroleum ether, Hexane and Water (aqueous extract)

Sr. no.	Solvent used	Stem Extract		Root Extract	
1	Petroleum ether	0.860	(Greenish colour)	1.927	(Greenish yellow colour)
2	Hexane	0.962	(Yellow colour)	1.829	(Yellow colour)
3	Distilled water	2.221	(Brownish colour)	1.396	(Dark brown colour)

3.2. Phytochemical screening and quantitative estimation of alkaloids, saponin and flavonoids:

Stem and root of *Moullavaspicata* were analysed for phytochemicals (Table 2) by using standardized phytochemical screening method. All the extract (aqueous, hexane and petroleum ether) shows the presence of all common phytochemicals (tannins, saponins, flavonoids, alkaloid, steroids, terpenoids and phenols). Quinones were detected in all extract of stem and root except aqueous extract whereas glycosides was observed in only aqueous extract of root. This screening results urged us to study quantitative analysis of major phytochemicals i.e. alkaloids, saponin and flavonoids (Table 3).

Table 2: Phytochemical screening of Stem and root extract (aqueous, hexane and petroleum ether) of *Moullavaspicata.*

Test	Stem			Root		
	Aqueous Extract	Hexane	Petroleum Ether	Aqueous extract	Hexane	Petroleum Ether
Tannins	+	+	+	+	-	-
Saponins	+	+	+	+	+	-
Flavonoids	+	-	+	+	-	-
Alkaloid	+	+	+	+	-	-
Quinones	+	+	+	-	+	+
Glycosides	-	-	-	+	-	-
Steroids	+	+	+	-	-	-
Terpenoids	+	+	+	+	+	+
Phenol	+	+	+	+	+	+
Anthocyanim & B-cyanin	+	-	-	-	-	-
Carbohydrates	+	+	+	-	+	+
Cardiac Glysosids	-			-	+	+

Presence is indicated by + sign and absence by – sign.

Table 3: Quantitative analysis of total alkaloid, saponins and flavonoid from *Moullavaspicata* root and stem extract.

Sr. No	Test	Root	Stem
1.	Alkaloid	17	20
2.	Saponins	776	128
3.	Flavonoid	501	232

Amount present in miligram per gram of extract.

Table 4: Antimicrobial activity of aqueous, hexane and petroleum ether extract of *Moullavaspicata.*

Sr.No	Microorganism	Stem Extracts			Root Extracts		
		Aqueous	Hexane	Petroleum	Aqueous	Hexane	Petroleum
1.	*Aspergillusniger*	-	-	-	-	-	-
2.	*Candida albicans*	10 mm	-	-	-	-	-
3.	*Staphylococcus cerevesiae*	-	-	-	-	-	-
4.	*Escherichia coli*	-	-	-	-	-	-
5.	*Staphylococcus aureus*	3 mm	-	-	-	3 mm	3 mm

Figures (mm) in the table indicate the zone of clearance due to plant extract against microorganism

Phytochemicals such as saponins, terpenoids, flavonoids, tannins, steroids, and alkaloids have antiinflammatory effects (Liu, 2003; Manach et al., 1996; Latha et al., 1998; Akindele and Adeyemi, 2007; Orhan et al., 2007; Muruganandan et al., 2001). Cherian and Augusti (1995) reported hypoglycemic activities of some polycyclic glycosides, flavonoids, tannins, and alkaloids. Saponins possess hypocholesterolemic and antidiabetic properties (Rupasinghe et al.; 2003). However, Luo et al., (1999) have shown that terpenoids (mono, di and tri) decrease blood sugar level in animal studies. High molecular weight steroids and triterpenoids showed analgesic properties (Sayyah et al., 2004; Malairajan et al., 2006). The steroids and saponins are also responsible for central nervous system activities (Argal and Pathak, 2006). Biochemical and phytochemicals screening of the hexane, petroleum ether and aqueous extracts of root and stem of this plant used in this present study revealed that the crude extracts contained alkaloids, flavonoids, glycosides, phytosterols, saponins, steroids, tannins, and triterpenoids (Table 2). Further study can be helpful to explore its candidature as antimicrobial agent. The present investigation is an important step in developing plant based pesticides which are ecofriendly for the management of fungi and development of commercial formulation of botanicals. Further studies are needed to determine the chemical identity of the bioactive compounds responsible for the observed antifungal activity. Natural plant-derived fungicides may be a source of new alternative active compounds, in particular with antifungal activity.

3.3. Antimicrobial assay:

Aqueous, P.E. and hexane extracts were screened for antimicrobial activity (Fig. 1,2 and 3) against fungus (*Aspergillusniger*, *Candida albicans* and *Saccharomyces cerevesine*) and bacteria (*E.coli*, *Staphylococcus aureus*). Among them hexane and PE root extract were found to be more effective showing zone of inhibition against *S. aureus* (Table 2). However stem aqueous extract also showed moderate zone of inhibition against *candida albicans* (10 mm) and *S.aureous*(3mm).

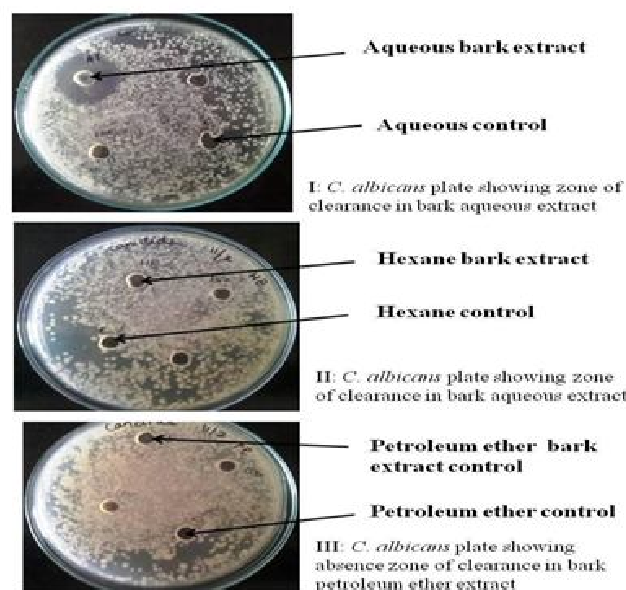

Fig.1. Inhibition zone produced by aqueous, hexane and petroleum ether stem extract Moullavaspicataagainst C. albicans.

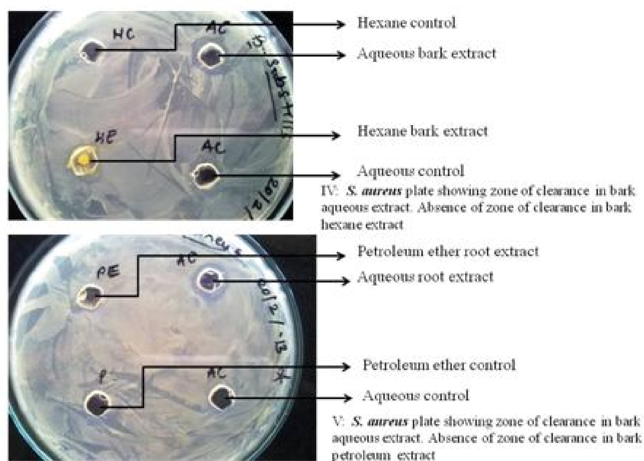

Fig.2. Inhibition zone produced by aqueous bark, hexane bark, petroleum ether root and aqueousextract of Moullavaspicata against S. aureus.

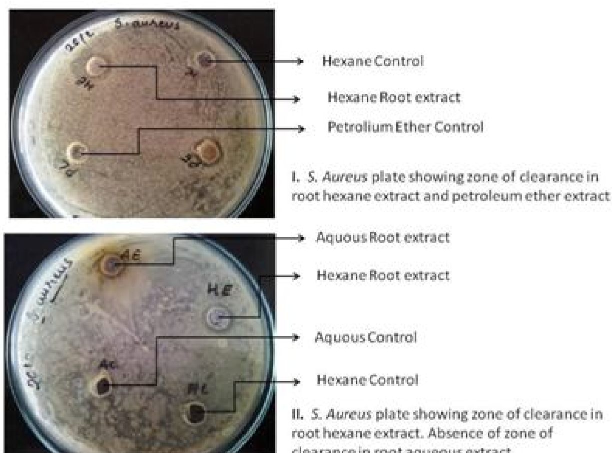

Fig.3. Inhibition zone produced by hexane root, petorlium ether root and aqueous root extract ofMoullavaspicataagainst S. aureus.

3.4. GC-MS analysis

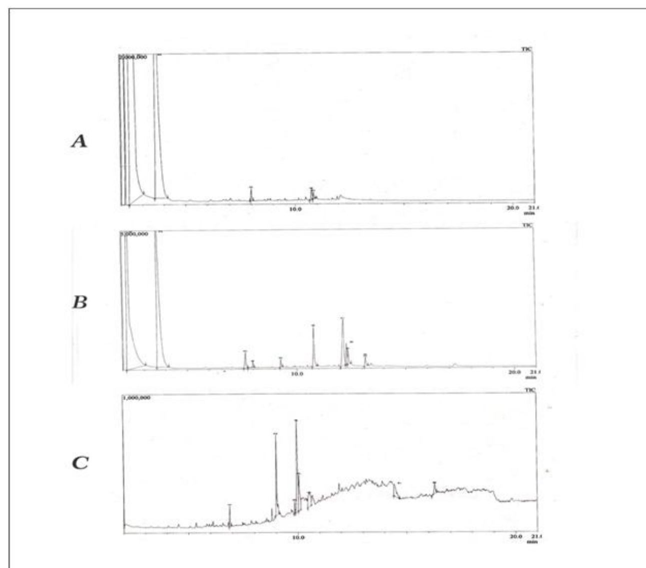

Fig.4.GC-MS analysis of hexane root, hexane bark and petroleum ether root extract of Moullavaspicata.

Hexane and PE root extract, due to their promising activity, were separately loaded in GC-MS system for chemical constituent analysis. Result revealed that both extract contain a common component i.e. diethyl phthalate. Specially pentane 2,3-dimethyl, hexane, nonadeanoic acid and dibutyl phthalate were detected in hexane root extract. However, n-hexadecanoic acid, octadecanoic acid methyl ester, oleic acid, 9-octadecenal and stigmas tan 3,5-diene were resolved completely from PE extract of root (Fig 4 A,B).

Chromatogram of hexane root (A) contain compounds Pentane,2,3-dimethyl (Peak-1), Hexane (Peck-2), Diethyl Phthalate (Peak-3), Nonadecaoic acid (Peak-4) and dibutyl phthalate (peak-5); hexane bark extract (B) contain compounds Pentafluoropropionic acid, heptyl ester (Peak -1), Hydroxylamine, O-(2-methylpropyl)- (Peak -2), Undecanoic acid (Peak -3), Phthalic acid, ethyl isopropyl ester (Peak -4), Tetradecanoic acid (Peak -5), n-Hexadecanoic acid 7 (Peak -6), Oleic acid (Peak -7), Oleic acid (Peak -8), Nonadecanoic acid (Peak -9), Z,Z,Z-4,6,9-8 Nonadecatriene (Peak -10) and Petroleum ether root (C) extract contain compounds Diethyl 9 Phthalate (Peak-1), n-Hexadecanoic acid (Peak-2), Octadecanoic acid, methyl ester (Peak-3), 10 Oleic acid (Peak-4), 9-Octadecenal (Peak-5), Stigmastan-3,5-diene (Peak-5).Pentane 2,3-diethyl was found in excess as compared to diethyl phthalate, nonagecanoic acid and dibutyl phthalate. Whereas nonadecanoic acid (peak 4) or Nonadecyclic acid (19-carbon long-chain saturated fatty acid) and dibutyl phthalate (peak 5) were not resolved. However, in PE root extract oleic acid (peak 4), n-Hexadecanoic acid (peak 2) were found in excess as compared to other components. (Fig 4B).

Several fragments of components were appeared on column except stigmastan-3,5diene and their retention time was from 11 to 20 min. Hexane extract led to identification of ten compounds from stem of this plant. The main constituents were pentafluoropropionic acid,Hydroxylamine, O-(2-methylpropyl)-, Undecanoic acid, Phthalic acid-ethyl isopropyl ester, Tetradecanoic acid, n-Hexadecanoic acid, Oleic acid (I), Oleic acid (II), Nonadecanoic acid, Z,Z,Z-4,6,9-Nonadecatriene. Synthetic structural analogs of n-Hexadecanoic acid are 3-ketohexadecanoic acid and 3-hexadecynoic acid are inhibitors of fatty acid biosynthesis (Carballeiraa et. al., 2006). Detected n-hexadecanoic acid from PE extract of bark may act as these compounds, therefore it was inhibit fungial growth showing zone of inhibition. Previous study related to structural analog of n-hexadecanoic acid i.e. 2,6-hexadecadiynoic acid displayed the strong antifungal activity against both the fluconazole resistant *Candida albicans* strains ATCC 14053 and ATCC 60193 (MIC = 11 μM) and against *Cryptococcus neoformans* ATCC 66031 (MIC < 5.7 μM) (Carballeiraa et. al., 2006). This report conclude that, antifungal activity of studied compound is depends on chemical structure of studied structural analog and fungal strain. In conclusion, 2,6- hexadecadiynoic acid exhibited the best fungitoxicity profile compared to other analogues. With respect to the toxicity of the 2-alkynoic acids it was recently demonstrated that 2-alkynoic acids could be used specifically against bacteria without toxicity to their host due to the fact that the FASI complex in microsomal systems is not inhibited by the 2-alkynoic acids (Morbidoni, 2006). In addition, it was previously reported that despite the fact that the 2- hexadecynoic acid inhibits the growth of HeLa cells, the simultaneous addition of palmitic acid to the culture medium reverses the growth inhibition observed in HeLa cells, thus implying that toxicity would not occur in animals consuming a normal diet (Konthikamee et al. 1982). Inhibition of the biosynthesis of the fungal fatty acids as shown for the 2-hexadecynoic acid in intact animals (Wood R and Lee T , 1981). Likewise, also analogous to the suspected mechanism of action for the 6-nonadecynoic acid, the 2,6-diynoic FA could also inhibit sphingolipid biosynthesis in these fungi (Li XC, 2003).

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