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BIOCHEMICAL STANDADRDIZATION OF MURRAYA KOENIGII

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ABSTRACT

The current research work proves the significance of herbal drugs in recent days, and it also provides the knowledge of safe use of drugs & their efficacy. Few standard parameters are used in this research for the standardization such as macroscopic and physico- chemical parameters, such as ash value, extractive values with different solvents, extraction of oil with its characterization & for evaluating efficacy of crude extracts Pharmacological activity has been performed such as antifungal activity.

INTRODUCTION

Description of *Murraya koenigii*

Phytography

The plant is a spreading shrub or small tree **(Fig. 1A)**. The main stem is dark green to brownish, with numerous dots on it. The bark can be peeled off longitudinally, exposing the white wood underneath. **(**Kirtikar et al. 1993) The girth of the main stem is 16 cm. Leaves**, (Fig.1B)** estipulate, bipinnately compound, 30 cm long, each bearing 24 leaflets, having reticulate venation; leaflets, lanceolate, 4.9 cm long, 1.8 cm broad, having 0.5-cm long petiole (Roy et al. 1977).

Fruits, round to oblong, 1.4 to 1.6 cm long, 1 to 1.2 cm in diameter **(Fig. 1A)**; fully ripe fruits, black with a very shining surface. Seed, one in each fruit, 11 mm long, 8 mm in diameter, color spinach green. Flowers are bisexual, white, funnel-shaped, sweetly scented,

stalked, complete, bracteates, regular, actinomorphic, pentamerous, and hypogynous (Khosa et al. 1997).

The average diameter of a fully opened flower is 1.12 cm. Inflorescence bears 60 to 90 flowers with calyx 5-lobed, persistent, inferior,green; corolla, white, polypetalous, inferior, with 5 petals, lanceolate, length, 5mm; androecium, polyandrous, inferior, with 10 stamens, arranged into circlesof five each; smaller stamens**(Fig.1B)**;4 mm. long whereas the longer ones, 5 to 6mm; gynoecium, 5 to 6 mm long; stigma, bright, sticky; style, short; ovary,superior (Khosa et al. 1997)

PHENOLOGY

Flowering and fruiting occurs between December to July.

Distribution

Murraya koenigii (L.) is an aromatic more or less deciduous shrub or a small tree up to 6 m in height, found throughout *India* up to an altitude of 1,500 m 1655 m, It abundantly occurs along the outer Himalayas, Assam, Andaman Islands, Maharashtra, Tamil Nadu, Andhra Pradesh and in the forests of Western Ghats in Karnataka more commonly in forest often as gregarious under-growths. It is cultivated for its aromatic leaves in south East Asia and Australia, Upper and Lower Burma **(**Anonymous et al. 1998).

Ecology and Cultivation

The plant grow best in tropical and sub-tropical climates in sunny to semi-shaded locations, though they can sustain in other climates by moving pots to warm protected areas in winter and maintaining humid conditions in areas where summers are hot and dry. They are very frost sensitive. Soil needs to be enriched with lots of organic material and be well drained. Seeds germinate readily (Prajapati et al. 2003**).** Almost every part of this plant has a strong characteristic odour.

Folklore and Traditional Uses

The bark and roots

It is used as a stimulant by the physicians. They are also used externally to cure eruptions and the bites of poisonous animals.

Green leaves

They are stated to be eaten raw for curing dysentery, and the infusion of the washed leaves stops vomiting. Curry leaves are also used in calcium deficiency. It has Vitamin A, Vitamin B, Vitamin C, Vitamin B2, Calcium and iron in plenty. Its nutritional value benefits both the

young and the old alike. Women who suffer from calcium deficiency, osteoporosis etc can find an ideal natural calcium supplement in curry leaves. Fresh juice of curry leaves, with lime juice and sugar, is an effective medicine in the treatment of morning sickness, nausea and Curry leaves can be used with effective result to treat burn, bruises and skin eruption. Cataract development can be prevented by using fresh juice of curry leaves. Kidney pain can be cured by using juice of root of *Murraya koenigii*. It can be used in preventing premature graying of hair.

Table.1 Plant profile of *Murraya koengii* (L.)

Botanical name	*Murraya koengii*	Kirtikar et al. 1993
Synonyms	*Murraya koengii, Mitha neem, Curry patta*	
Family	*Rutaceae*	

Table.2 Vernacular names of *Murraya Koengii* (Kirtikar et al. 1993)

English	*Murraya koengii*
Sanskrit	*Krishna nimbi*
Hindi	*Kathnim, Mitha neem, Curry patta*
Gujarati	*Goranimb, Kadhilimbdo*

A

B

Fig.1 A and B Whole Plant of *Murraya koenigii* and Flowers along with Fruits.

Table.3 Taxonomical classification (Kirtikar et al. 1993)

Kingdom	*Plantae*
Division	*Magnoliophyta*
Class	*Magnoliopsida*

Order	*Sapindales*
Family	*Rutaceae*
Genus	*Murraya*
Species	*koenigii*

EXPERIMENTAL

MATERIAL & METHODS

PLANT MATERIALS

Collection and Identification of Plant Material

Plant *Murraya koenigii* was collected from Kheri Bawli Delhi India. The plants were identified by Prof. Dr.Anju Pal, Horticulture department, panthnagar university, Panthnagar, Uttarnchal, India.

Solvents and chemicals

All the solvents used for extraction and isolation like methanol, , chloroform, n-hexane, ethyl ecetate, ethanol, proponol, n- butanol Vanillin, silica gel (70-230 mesh) and TLC aluuminium sheets 20 x 20 cm, Silica gel 60 F_{254}. , were imported from Merck KgaA Darmstadt Germany. Sephadex LH-20 25-100|jm FlukaChemie GmbH (9041-37-6).

Macroscopic Identification

Thin section were made with the help of blade, stained and mounted following the usual plant micro-techniques. For the study of isolated cells and tissues, small pieces of leaves, roots, stem, were taken. Washed and mounted in glycerine. The anatomical sketches were made with digital camera.

Quantitative Leaf Microscopy

Quantitative leaf microscopy to determine palisade ratio, stomata number, stomata index, vein -

islet number and veinlet termination number were carried out on epidermal strips

Preliminary Screening of Phytochemicals

The preliminary phytochemical studies were performed for testing the different chemical groups present the drugs 10% (w/v) solution of extract was taken unless otherwise mentioned in the respective individual test. The chemical group test were performed and the result are

shown in tables. General screening of various extracts of the plant material was carried out for qualitative determination of the groups of organic compounds present in them.

Alkaliod

Test for alkaloids are following:

Dragendroff's test

Dissolve a few mg of alcoholic extract of the in 5 ml of distilled water, add 2 M hydrochloric acid until an acid reaction occurs, then add 1 ml of Dragendroff's reagents, *orange or orange-red ppt is produced immediately.*

Hager's test

To 1 ml of alcoholic extract of the drug taken in test tube, add a few drops of Hager's reagent. Formation of yellow ppt confirms the presence of alkaloids.

Wagner's test

Acidify 1 ml of alcoholic extract of the drug with1.5% v/v of hydrochloric acid and add a few drops of Wagner's reagent. A yellow or brown ppt is formed.

Mayer's reagent

Add a few drops of mayer's reagent to 1 ml of alcoholic extracts of the drug. White or pale yellow ppt. is forme

Carbohydrates

Test for crbohydrates are following:

Anthrone test

To 2 ml of anthrone test solution, add 0.5 ml of alcoholic extracts of the drug. A green or blue color indicates the presence of carbohydrates.

Benedict's test

To 0.5 ml of alcoholic extracts of the drug add 5 ml of Benedict's solution and boil for 5 mins. Formation of a brick red coloured ppt is due to presence of carbohydrates.

Fehling's test

To 2 ml of alcoholic extracts of the drug add 1 ml of the mixture of equal parts of fehling's solution 'A' and 'B' then boil the contents of the test tube for few mins. A red or brick red ppt is formed.

Molisch's test

In test tube containing 2 ml of alcoholic extracts of the drug add 2 drops of a freshly prepared 20% alcoholic solution of β napthol mix poured 2 ml of concsulphuric acid so as to

form a layer below the mixture. Carbohydrates, if present, produce a red- violet ring, which disappears on the addition of an excess of alkali solution.

Flavonoids

Test for flavonoids are following:

Shinoda's test

In a test tube containing 0.5 ml of alcoholic extract of the drug, add 5-10 drops of dilute hydrochloric acid followed by a small piece of magnesium. In the presence of flavonoids a pink, reddish pink or brown colour is produced.

Triterpenoids

Test for flavonoids are following:

Liebermann-Burchard's test

Add 2 ml of acetic anhydride soloution to1 ml of alcoholic extracts drug in chloroform followed by 1 ml of con sulphuric acid.A violet color coloured ring is formed shows the presence.

Saponins

In a test tube containing about 5 ml of an alcoholic extracts of the drug add a drop of sodium bicarbonate solution, shake the mixture vigorously and leave for 3 mins. Honeycomb like froth is formed.

Steroids

Test for steroids are following:

Liebermann-Burchard's test

Add 2 ml of acetic anhydride soloution to 1 ml of alcoholic extracts of the drug in chloroform followed by 1 ml of con sulphuric acid. A greenish colour is developed which turns to blue.

Salkowaski reaction

Add 1ml of conc. Sulphuricacid to 2 ml of alcoholic extracts of the drug carefully, from the side of the test tube. A red colour is produced in the chloroform layer.

Tannins

Test for tanins are following:

To 1-2 ml of plant alcoholic extracts extract, add a few drops of 5% $FeCl_3$ solution was added. A green colour indicates the presence of gallotannins while brown colour tannins.

Starch

Test for starch are following:

Dissolve 0.015g of iodine and 0.075g of potassium Iodide in 5 ml of distilled water and add 2-3 ml of an alcoholic extracts of drug. A blue color is produced.

Extraction & Characterization of Volatile and Non-Polar Component of *Murraya koenigii*

Plant material

Plant *Murraya koenigii* was collected from Kheri Bawli Delhi India. The plants were identified by Prof. Dr.Anju Pal, Horticulture department, panthnagar university, Panthnagar, Uttarnchal, India.

Extraction and Isolation of Oil

The aerial parts of fresh plants were subjected to hydrodistillation in a Clevenger type apparatus (1 kg each) for 3 h. The distillate was saturated with NaCl and the oil was extracted with *n*-hexane and dichloromethane. The solvent phase was then dried over anhydrous Na_2SO_4 and then the solvent distilled off at 35ºC under vacuum using rotary vacuum evaporator (Buchi, Switzerland). The oil yield of plant material was 0.063% (v/w, fresh wt basis). The oil samples were stored at -20ºC until analyzed.

Instrumentation and GC Conditions

A PerkinElmer Autosystem XL gas chromatograph with flame ionization detector (GC-FID) was used, system fitted with a bonded; poly (5% diphenyl/95% dimethylsiloxane), column, EQUITY–5 (60 m x 0.32 mm, film thickness 0.25 µm, SUPELCO, USA). The column temperature ranged from70-250ºC, at 3ºC/min and 250-320ºC, at 6ºC/min, with a final hold time of 5 min, using H_2 as carrier gas at 10 psiconstant pressure, a split ratio of 1:50, an injection size of 0.03 µL neat, and injector and detector (FID)temperatures of 280ºC and 300ºC, respectively.

GC/MS utilized a PerkinElmer Autosystem XL gas chromatograph interfaced with a Turbomass Quadrupole Massspectrometer detector (GC-MS) fitted with a bonded; poly(5% diphenyl/95% dimethylsiloxane), column, fused silica capillary column EQUITY–5 (60 m x 0.32 mm, film thickness 0.25 µm, SUPELCO, USA). The column temperature of 70ºC-300ºC was programmed at a rate of 3.0ºC /min, with a hold time of 10 min. The oven temperature program was the same in as in GC while the injector temperature was 270°C, transfer line and ion source temperatures were 300°C, injection size 0.03µL neat, split ratio 1:50 using He

as carrier gas at 10 psi constant pressure. MS were taken at 70 eV with a mass range of m/z 40-450. Characterization was achieved on the basis of retention time, Kovats Index, literature reported retention index. Using a homologous series of *n*-alkanes (C_8-C_{25} hydrocarbons, Polyscience Corp. Niles IL), co-injection with standards (Sigma Aldrich), mass spectra library search, and by comparing with the mass spectral literature data. The relative amounts of individual components were calculated based on GC peak areas without using correction factors.

PHARMACOLOGICAL ACTIVITY

Antifungal Assay

Antifungal activity was carried out following agar tube dilution protocol [Paxton, 1991] Methanolic extract and various fractions were used in dose of 24 mg/ml and purecompounds 12 mg/ml of sterile DMSO, provided as stock solution. The agar dilutionmethod is the most convenient method for routine testing of samples such as plant extracts.The method is suitable for testing non- sterile plant extracts, because aerobic organisms donot develop well under the solidified agar. However, the occasional culture that develops onthe surface of the agar can be easily recognized. Non-polar extracts, essential oils,suspensions of solids or emulsions and antimicrobial substances, which do not diffuse through agar media, can be tested directly by incorporating them with the agar media as ifthey were aqueous solutions. This method has an advantage that unlike the diffusionmethod, no concentration gradient occurs during the testing procedure. Moreover, several different test microorganisms may be tested simultaneously on the same dilution, which makes the agar dilution method very efficient. Antifungal activity was carried out againstclinical specimen of human pathogens namely *Candida albicans*(ATCC 2091), *Candidaglabarata* (ATCC 90030), *Aspergillus flavus* (ATCC 32611) *Trichophytonlongifusis*(clinical isolate),*Mycosporumcanis* (ATCC 11622) and *Fusariumsolani*(ATCC 11712).

Table.4 Description of Sabouraud Dextrose Agar (SDA)

Sabouraud Dextrose Agar (SDA)	
Formula	**G/Litre**
Mycological peptone	10.0
Glucose	40.0

Agar	15.0
pH	5.6+0.2

(15 g) was dissolved in distilled water and volume was made to 1 liter, the mixture washeated for at least 10 minutes and then sterilized in autoclave (15 lbs/in2 pressure and at121°C temperature for 15 minutes). The culture of organisms was maintained on SabouraudDextrose Agar (SDA). Sabouraud Dextrose Agar (SDA), 4 ml was distributed into attachedcap tubes, that were then placed in autoclave at 121 0C for fifteen minutes andthentemperature was brought to 50 0C. The stock solution (66.6 µl) was then added to nonsolidified SDA media, giving final concentration of 400 µg of extract per ml of SDA. Tubeswere then left to solidifying in angled pose at room temperature. Every tube was implantedwith a piece of inoculums (4 mm diameter), detached from seven days old culture ofrespective fungi. For non mycelial growth, an agar surface band was used. Resistance offungal growth was recorded after seven days of incubation at 28+1 0C. Media supplementedwith DMSO and standard anti-fungal drugs are used as +veand -ive control correspondingly.Miconazolewas used as reference antifungal drug for all pathogens except*C.albicans*forwhich Amphotericin-B was used as a reference drug. Growth in medium containing extractswas calculated by determining linear growth in mm and then percent growth inhibition wasmeasured with standard to -ve control using formula.

$$\%\ inhibition = \frac{Growth\ in\ sample\ tube(mm)}{Growth\ in\ control\ tube(mm)} \times 100$$

Table.5 Description for calculating Antifungal Assay

Criteria for DeterminingAntifungal Assay		
S.No	Percent Inhibition	Activity
1.	30-40	Low
2.	50-60	Moderate
3.	61-70	Good
4.	Above 70	Significant

RESULTS AND DISCUSSION

Macroscopic Characters of Stem of *Murraya koenigii*

Stem

Murraya koenigii is an aromatic and small tree up to 6 m in height and 15-40 cm in diameter. The young stems are green in color with sweet aromatic odor and characteristic taste. The outer surface is smooth, soft and glabrous. The mature stems of *Murraya koenigii* are dark brown (unpeeled) and Cremish brown (peeled) in color with slight aromatic odor and characteristic taste. The outer surface is smooth and hard. The fracture of bark is splintery.

Leaf

Leaves are compound, imparipinnate, petiolate, exstipulate, rachis 11 to 20 cm long; leaflets 11 to 25, shortly petiolulate, arranged alternately on the rachis; lower pairs comparatively smaller in size, obliquely ovate, 2 to 5 cm in length and 1 to 2.5 cm in width, tip acute to obtuse, margin crenate-dentate, glabrous adaxially and pubescent abaxially with interspersed gland dots; main vein one and lateral veins 14 to 20 pairs; odour; taste, acrid.

Table.6 Quantitative Leaf Microscopy of *Murraya koenigii.*

Parameters	**Range**	**Mean***
Palisade Ratio	11-14	12.85 ± 0.35
Stomatal Number Upper surface	0	0
Stomatal Number Lower surface	67-82	66.31 ± 6.81
Stomatal Index Upper surface	0	0
Stomatal Index Lower surface	13.47-15.42	14.68 ± 0.22
Vein islet number	12-15	13.64 ± 0.42
Veinlet Termination Number	9-12	13.62 ± 0.29

*** Mean value of 10 counts**

Table.7 Phytochemical Screenig of *Murraya Koenigii*

Phytochemical tests	***Murraya koenigii* Leaf Extracts**			
Active constituents	**MCR**	**MPE**	**MAC**	**MME**
Alkalodids	+++	++	++	+

Flavonoids	++	++	+++	+
Saponins	_	+++	+++	++
Tannins	+++	+++	+++	++
Steroids	+++	+++	+++	+++
Cardiac Glycosides	+	++	++	+++
Proteins	++	+++	++	+
Resins	_	_	_	_
Starch	_	_	_	_
Triterpenoids	+	++	++	+
Carbohydrates	+++	++	+++	+

(-): No presence, (+): Less presence, (++): Moderate Presence,(+++): High presence, MCR: Crude powder, MPE: Petroleum ether extract, MAC: Acetone extract, MME: Methanol Extract, Common in MPE and MME. the Constituents can be further isolated and purified to find its potency for biological activities.[M= *Murraya koenigii]*

VOLATILE AND NON-POLAR COMPONENT OF *MURRAYA KOENIGII*

The essential oil obtained by steam distillation was anlaysied by GC-FID and GC-MS. The compounds were charaterised by comparision with NIST-Wiley library of mass as well as on the basis of kovate indices. The compound characterized are summarized in **Table.8**

α-Humulene, trans β-Caryophyllene, α- Pinene, β Phellanderene were the major components in **4.87, 27.2** and **19.0318.22 %** respectively

Table.8 Chemical Composition of *Murraya Koenigii* From Vegetative and Flowering Plant Oil.

Chemical Composition of *Murraya Koenigii* From Vegetative and Flowering Plant Oil.					
S.No	Compound name*	RI	LRI	% Content in Aerial part	Mode,of identification

1	α Humulene	1448	1452	4.87	MS
2	trans-**β**-Caryophyllene	1417	1415	27.2	LRI
3	Bicyclogermacrene	1490	1492	5.23	LRI
4	α -Pinene	932	931	19.03	MS
5	**β** Phellanderne	1025	1026	18.22	LRI
6	α -Amorphene	1483	1481	1.88	MS
7	**α**-Phellandrene	1002	1003	1.87	MS
8	**β**-Pinene	974	973	4.03	MS
9	**β**Myrcene	988	987	0.83	MS
10	Camphor	1141	1143	1.28	MS

RI on Equity-5 columns using a homologous series of n-alkanes (C9-C28 hydrocarbons, Polyscience Corp. Niles IL); LRI-RI reported in literature MS-mass spectrum, STD=Sigma Standard, t=trace <0.1%, * tentatively identified.

Antifungal Assay

Antifungal activity of methanolic crude extract and different portions were evaluated against fungal strains included Trichophyton longifusus (clinical isolate), Candida albicans ATCC2091, Aspergillus flavus ATCC 32611, Microspoum canis ATCC 11622, Fusarium solani ATCC 11712 and Candida glaberata ATCC 90030. They were maintained on agar slant at 4o C. The strains were activated at 37^0C for 24 hrs on nutrient agar (NA) or Sabouraud glucose agar (SGA) respectively for fungi, prior to any screening. Growth inhibition was presented in percent in comparison to standard drugs. Amphotericin-B was employed as representative drug against Aspergillus flavus while miconazole was used as standard drug against rest of fungal strains table. The dose was given in a single concentration (400µg/ml). The *Murraya Koenigii* crude extract (MK-1), n-hexane (MK-2), Chloroform (MK-3), Butanol fraction (MK-5) and aqueous fraction (MK-6) exhibited the inhibitory activities having region of reticence in mm against these strains (Table. 28). Crude extract of *Murraya Koenigii* exhibited antifungal activity against *Trichophyton*

longifusus,Aspergillus flavus, *Microsporum canis* and *Fusarium solani* by inhibiting 49 %,52 %,54 % and 42 % respectively. Similarly n-hexane fraction inhibited growth by 60%, 87% and 78% of *Aspergillus flavus*, *Mycosporum canis* and *Fusarium solani* respectively. While the chloroform fraction showed the inhibitory effect against *Aspergillus flavus, Mycosporum canis* and *Fusarium solani* by 66%, 80% and 70% respectively. Ethyl acetate produced inhibitory effect against *Trichophyton longifusus*, *Mycosporum canis*, *Aspergillus flavus* and *Fusarium solani*of test organism in patron of 50 %, 55 %, 08%, 60% and 31 % respectively. The butanol fraction exhibit the inhibition against Aspergillus flavus, Mycosporum canis and Fusarium solani. 22 %, 66 %and 40 % respectivel. While in case of aqueous fraction the % inhibition against the test organisms Trichophyton longifusus, Aspergillus flavus, Mycosporum canis and Fusarium solani was 30 %, 56 %, 51 % and 68 %each . However, all the fractions as well as crude methanolic extract illustrated no activity against Candida albicans and Candida glaberata.

Table. 9 Result of antifungal assay of crude extract and fractions

S.No.	**Result of antifungal assay of crude extract and fractions**							
	Fungal Strain	**% Inhibition**						
		MK-1	**MK-2**	**MK-3**	**MK-4**	**MK-5**	**MK-6**	**Standerd drug**
1	*T.* longifusis	49	-	-	50	-	30	Miconazole
2	C. albicans	-	-	-	55	-	-	Miconazole
3	A. flavus	52	60	66	08	22	56	Amphotericin-B
4	M. canis	54	87	80	60	66	51	Miconazole
5	F. solani	42	78	70	31	40	68	Miconazole
6	C. glabarata	-	-	-	-	-	-	Miconazole

T. longifusis: Trichophyton longifusus ***M. canis: Mycosporum canis***

C.albicans: Candida albicans ***F solani: Fusarium solani***

A. flavus:Aspergillus flavus ***C. glabarata: Candida glaberata***

MK-1: Crude extract **MK-5: n-BuOH fraction**

MK-2: *n*-hexane fraction **MK-6: H2O fraction**

MK-3: $CHCl_3$ fraction **STD: Imipenum.**

% inhibition of fungal growth = 100 – linear growth in test (mm) X 100

Linear growth in control (mm)

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