Evaluation of the Phytochemical, Antioxidant and Anti-microbial Activities obtained from the Methanolic Leaf Extracts of *Melia dubia* Cav.

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ABSTRACT *Melia dubia* Cav. is a native species of tree to India and a sub canopy tree species naturally found in the tropical moist deciduous and semi-evergreen forest of south Indian region. The leaves of *M. dubia* samples collected from the Chikkaballapura (MDL1) and Bengaluru (MDL2) region were screened for the detection of phytochemical contents and for the determination of antioxidants such as ABTS Radical Scavenging Assay, Total Reducing Power Assay, Hydrogen peroxide radical scavenging activity and their evaluation of anti-microbial activity. Phytochemical analysis of leaf extract of *M. dubia* revealed that the presence of significant plant secondary metabolites contributes to its anti-microbial and antioxidant activity. Thus, *M. dubia* can be used as a potential source for development of potential anti-microbial drug of botanical origin comparable to synthetic pesticides. Phytochemicals contents present in the leaf extracts are capable of rendering health benefits, thereby demonstrating that *M. dubia* can be a probable medicinal plant for human mankind.

KEYWORDS: Antioxidants; Anti-microbial activity; Phytochemicals; Secondary metabolites.
Received 20 September 2020 **Revised** 23 October 2020 **Accepted** 1 November 2020 **In press** 3 November 2020 **Online** 2 January 2021

Original Article

INTRODUCTION

Meliaceae (Magnoliopsida: Sapindales) is a large family, mainly of tropical trees producing many commercially important and well-known choice hardwoods (Styles, 1971). Among the 12 species of genus *Melia*, four of them such as *M. dubia*, *M. azadirach*, *M. compacta* and *M. composita* are found in India (Gupta, 1993). Among these, *M. dubia* (earlier known as *M. composita*) commonly known as Malabar Neem because its wood is very popular in southern states of India for its fast growth and wide adaptability in diverse edaphic and climatic conditions. The world agroforestry centre has identified *M. dubia*, *M. toosendan*, *M. compacta* and *M. volkensi*, as very useful in tree domestication programs.

Melia dubia is a sub canopy tree species naturally found in the tropical moist deciduous and semi-evergreen forest of South and Central Western Ghats, Eastern Ghats and North East India at altitudes of 1,500-1,800 m. It is widely naturalized in Europe, America and Australia (Mabberley, 1984). *M. dubia* is a large deciduous and native tree species to India, lately one of the most extensively planted tree species in Southern India. *M. dubia* has shown great potential for the best management in terms of secondary plant chemistry.

Every part of the *M. dubia* plant is being used as traditional herbal medicines such as anthelmintics, treatment of leprosy, eczema, asthma, malaria, fevers and venereal diseases (Govindachari, 1992), as well as cholelithiasis, acariasis and pain (Kokwaro, 1976). Fruits of *M. dubia* are considered to be important in colic and skin diseases and also as anthelmintic (Purushothaman *et al.*, 1984). It is well known as rich and valuable source of bioactive limonoids with highly oxygenated, modified terpenoids and have recently attracted attention (Awang *et al.*, 2007) because compounds belonging to this group have exhibited a range of biological activities such as

insecticidal and insect antifeedant especially on some of the forest insect pests and growth regulating activity on insects as well as antibacterial, antifungal, antimalarial, anticancer, antiviral and a number of other pharmacological activities on humans (Nakagawa *et al.*, 2001; Endo *et al.*, 2002; Koul *et al.*, 2004).

Fruit of the plant is bitter and is considered anti-helminthic. Total fruit extracts are found to be most effective as an anti-diabetic agent when tested on mice for its efficacy as a potential hypo-glycaemic drug (Khadse & Kakde, 2014). Plant leaf is a good source of essential oil with a volatile monoterpene camphene (Nagalakshmi *et al.*, 2001) and shown good anti-microbial activity inhibited 88% of skin pathogens (Gerige & Ramjaneyulu, 2007). The leaf and bark extracts exhibited anti-feedant, insecticidal, anti-bacterial, anti-viral and anti-fungal property (Mikolajczak & Reed, 1987; Susheela *et al.*, 2008; and Malarvannan *et al.*, 2009). Presence of shikimic acid in the oil forms the key precursor for the synthesis of *Tami flu* which makes it the only drug against *avian flu* caused by the *H5N1 virus* in plant leaf extract (Raghavendra *et al.*, 2009).

Methanol extracts from twigs and bark of *M. dubia* have anti-feedant and growth inhibitory activities on many pest and insect larval instars and the allelochemical toosendanin was isolated from this fraction contain is the active principle Tetranortriterpenoids (Purushothaman *et al.*, 1984) and monoterpenes (Nagalakshmi *et al.*, 2001) are its major constituents present in the extract and reported to be toxic to pest and insects larvae (Opender *et al.*, 2000). Considering all of these facts, the present study was designed to investigate a comprehensive phytochemical, antioxidant and anti-microbial profiling of *M. dubia*.

METHODOLOGY

Sample Collection and Methanolic Leaf Extraction

The leaves of *M. dubia* plants were collected from the Chikkaballapur (MDL1) and Bengaluru (MDL2) region. Plant leaves were washed thoroughly in running tap water to remove soil particles and other adhered debris, then shade dried for 14 days and ground well into fine powder. Powdered materials were stored in air tight container until the time of use. 50g of this powdered material were soaked in 100ml of methanol and kept at room temperature for 12h and kept at shaker for 3h. Samples were filtered and used for phytochemical screening and excess filtrate was filtered through a single layer of muslin cloth, and then final filtrate was collected by passing it through a Whatman filter paper no. 1 in a funnel. The filtrate was evaporated to dryness and thus, the crude extract of *M. dubia* was obtained (Arekemase *et al.*, 2011; Selvamangai *et al.*, 2012).

Phytochemical analysis

M. dubia samples were screened phytochemicals using the following standard laboratory techniques (Harborne, 1992; Sofowara, 1993) which is given below in Table 1.

Anti-oxidant Assay

ABTS Radical Scavenging Assay is performed using the standard method followed by Auddy *et al.*, (2003). Percent inhibition and the IC_{50} values are calculated by using Graph pad prism. Total Reducing Power Assay is performed using the standard method followed by Benzie and Strain (1999). Hydrogen Peroxide Radical Scavenging Activity is implemented using the standard method followed by Halliwell *et al.*, (1987). Percentage inhibition of hydroxyl radicals is calculated by the formula as given below:

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test sample)}}{\text{Absorbance (control)}} \times 100$$

Table 1. Screening of the methanolic leaf extracts of *Melia dubia* samples for the phytochemicals content using standard techniques (Harborne, 1992; Sofowara, 1993)

Sl. No.	Phytochemicals	Protocols	Inference
1	**Alkaloids**	0.2ml of sample + 0.2ml of HCl + Dragendoff's reagent	Appearance of orange or red precipitate
2	**Carbohydrates**	0.2ml of sample + a few drops Molisch's reagent (α-napthol in alcohol) + 0.2 ml of Conc. H_2SO_4 along the sides of the test tube	Appearance of a purple colour ring
3	**Tannins**	0.2 ml of plant extract + 2 ml of water and heated on water bath for 10 min. Then mixture was filtered and ferric chloride was added to the filtrate and observed	Indication of dark green solution
4	**Terpenoids**	0.2 ml of plant extract + 0.2 ml of chloroform + Conc. H_2SO_4 added carefully to form a layer	Presence of reddish brown colour at the interface
5	**Glycosides**	0.2 ml of sample + 0.2 ml of chloroform + 0.2ml of acetic acid. Mixture was cooled on ice and then, Conc. H_2SO_4 added carefully	Colour change from violet to blue to green indicating the presence of steroidal nucleus
6	**Steroids**	0.2 ml of sample + 0.2 ml of chloroform + 0.2 ml of Conc. H_2SO_4	Appearance of red colour in the lower layer of chloroform
7	**Saponins**	0.5 g of extract + 5 ml of distilled water + shake vigorously and observe for a stable persistent froth. Then frothing was mixed with 3 drops of olive oil and shaken vigorously	Observe for the formation of an emulsion
8	**Flavonoids**	0.2 ml of plant extract + dilute sodium hydroxide solution + diluted hydrochloric acid	Observation of yellow solution which turn colourless later would indicate the presence of flavonoids.
9	**Protein**	0.2 ml of extract + 0.2 ml Conc. H_2SO_4.	After the appearance of white precipitate, the extract was boiled to get yellow precipitate which indicates the presence of proteins.
10	**Mucilage**	0.2ml of extract + 0.2ml of absolute alcohol and then allowed to try.	If the precipitation occurs then mucilage is present.
11	**Phenols**	1ml of aqueous extract + 2 ml of distilled water + a few drops of 10% aqueous ferric chloride solution	Formation of blue or green colour indicates the presence of phenols.

Evaluation of Anti-microbial activity of M. dubia

Anti-microbial activity for bacteria was determined by using the well diffusion technique culture methods. Anti-microbial activity of the leaf extracts namely, MDL1 and MDL2 were tested on *Escherichia coli* and *Streptococcus mutans*. Petri plates (with diameter of 90mm) containing 20mL Meuller-Hinton agar were seeded using cotton swab with 24h (old) culture of the microbial strains. 10mg was weighed and dissolved in 100µl of methanol to get a final concentration of 100mg/ml. Four gram of soya bean casein digest agar (Soya bean-casein digest agar mainly for bacteria) was weighed accurately and dissolved in 100ml of distilled water and autoclaved. 100µl Inoculum of test cultures were inoculated on this digest agar plates (90mm). Test compounds (25µl), and ciprofloxacin (25µl, 0.1mg/mL) were impregnated on 5mm wells on agar plates. Plates were incubated @ 35^0C for 24-48h and observe for zone of inhibition around the well.

RESULT AND DISCUSSION

Phytochemical analysis

Qualitative estimation of the phytochemicals conducted on *M. dubia* leaf extracts (MDL1) revealed the presence of alkaloids, phenolics, flavonoids, and tannins (Table 2), whereas *M. dubia* leaf extract (MDL2) revealed the presence of alkaloids, tannins, terpenoids, flavanoids and phenolics. The active principles of many drugs found in plants are known for secondary metabolites.

Table 2. Phytochemical analysis of the methanol extract of *M.dubia*

Sl. No.	Phytochemicals	Plant samples	
		MDL1	MDL2
1	Alkaloids	+	+
2	Carbohydrates	_	_
3	Tannins	+	+
4	Terpenoids	_	+
5	Glycosides	_	_
6	Steroids	_	_
7	Saponins	_	_
8	Flavanoids	+	+
9	Proteins	_	_
10	Mucilages	_	_
11	Phenolics	+	+

ABTS Anti-oxidant Assay

Table 3. ABTS radical scavenging activity of the methanol extract of *M. dubia*

Compound Name	Concentration (µg/ ml)	Absorbance (590 nm)	Inhibition (%)	IC_{50} (µg/ ml)
Control	0.0000	0.73	00.00	-
Quercitin	0.3125	0.72	01.44	05.96
	0.6250	0.72	02.13	
	1.2500	0.64	13.12	
	2.5000	0.47	35.48	
	5.0000	0.42	43.26	
	10.0000	0.08	89.30	
	20.0000	0.07	90.80	
MDL1	3.1250	0.71	02.73	116.40
	6.2500	0.68	07.79	
	12.5000	0.61	17.55	
	25.0000	0.60	18.57	
	50.0000	0.40	44.90	
	100.0000	0.27	62.99	
MDL2	3.1250	0.67	08.07	55.20
	6.2500	0.60	18.04	
	12.5000	0.58	20.33	
	25.0000	0.43	41.45	
	50.0000	0.19	73.99	
	100.0000	0.08	88.68	

Methanolic leaf extract of *M. dubia* were evaluated for antioxidant activity by using the ABTS, DPPH and H_2O_2 models. *MDL*1 and *MDL2* exhibited potent antioxidants in ABTS free radical assay as compared to standard Quercetin (Table 3). In ABTS assay, MDL1 exhibited IC_{50} value of 116.40 µg/ml and MDL2 showed IC_{50} values of 55.20µg/ml. IC_{50} Free radical scavenging assays using synthetic radicals of standard quercetin was found to be 5.96µg/ml. Biological radicals such as superoxide radical anions offer an easy and rapid way to screen herbal drugs, food and beverages for *in vitro* antioxidant activity. Antioxidants can play a protective role to inactivate harmful reactive oxygen species (Sudha *et al.*, 2011). Antioxidant activity of phenolic compounds is mainly attributed to their redox properties and allow them to act as reducing agents, hydrogen donor and quenchers of singlet oxygen (Rice-Evans *et al.*, 1997, Meda *et al.*, 2005). It reflects the ability of hydrogen donating antioxidant in the extract to scavenging the ABTS radical cation compared with that of

quercitin suggesting that the tested samples may prevent the *in vitro* formation of radical species related with oxidative stress. This play an important role in protecting against damage to membrane functions (Puertas *et al.*, 2005).

Total Reducing Power Assay

The amount of iron reduced and can be expressed as μmolar Fe^{2+} equivalents or relative to an antioxidant reference standard Vitamin C. Higher absorbance value indicates a stronger reducing power of the samples (Table 4). MDL1 and MDL2 extracts showed concentration-dependent reducing power. However, its reducing power at highest concentration at 1mg/ml is 392.69, 288.19, 254.06 and 148.38μg Vitamin C/g equivalent to standard respectively. Overall, all the tested samples have good antioxidant activity when compared to the standard.

Table 4. Ferric reducing antioxidant power activity of methanolic leaf extract of MDL1 and MDL2, and standard Vitamin C

Compound Name	Concentration (μg/ ml)	Absorbance (700 nm)	Total reducing power (mg VC/g)
Control	00	0.12	
Vitamin C	50	0.29	
	100	0.43	
	200	0.58	
MDL1	100	0.19	
	200	0.28	254.06
	400	0.40	
	800	0.56	
MDL2	100	0.17	
	200	0.20	148.38
	400	0.28	

Free radical scavenging potential of the extract was determined by DPPH assay. Since the DPPH assay is sensitive enough to detect natural compound at low concentration, it is extensively used for evaluating the free radical scavenging potential of natural antioxidants (Porto *et al.*, 2000). Scavenging activity of extracts could be related to the nature of phenolics and their hydrogen donating ability. Antioxidant activity of MDL1 was higher than that of MDL2. The IC_{50} value for each sample was determined graphically by plotting the percentage disappearance of DPPH as a function of the sample concentration. IC_{50} for MDL2 was 100 μg/ml which was higher than the positive quercetin (IC_{50} = 5.96μg/ml). Thus, DPPH is a stable free radical, hydrogen-donating and accepting an electron or hydrogen radical by reacting with antioxidants to become stable diamagnetic molecule (Blios, 1958; Soares *et al.*, 1997).

H_2O_2 Anti-oxidant Assay

MDL1 and MDL2 exhibited potent antioxidants in H_2O_2 free radical assay as compared to standard quercetin (Table 5). Hydroxy radicals are an extremely reactive free radical formed in biological system and has enhanced as highly damaged to almost every molecule found in living cells. These radicals have the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutation and cytotoxicity, and considered to be one of the quick initiators of lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids (Gordon, 1990).

Table 5. Hydrogen peroxide assay of standard gallic acid and methanolic leaf extract of *M. dubia* MDL1 and MDL2

Standard name	Concentration (µg/ ml)	Absorbance (532 nm)	Inhibition (%)	IC_{50} (µg/ ml)
Control	00	00.59	00.00	
Standard (Gallic acid)	10	08.10	08.10	**20.54**
	20	18.83	18.83	
	40	39.86	39.86	
	80	51.55	51.55	
	160	58.99	58.99	
	320	63.11	63.11	
MDL1	10	00.55	06.78	**180.80**
	20	00.51	13.56	
	40	00.47	20.34	
	80	00.40	32.20	
	160	00.37	37.29	
	320	00.25	57.63	
MDL2	10	00.56	05.08	**29.63**
	20	00.52	11.86	
	40	00.48	18.64	
	80	00.41	30.51	
	160	00.33	44.07	
	320	00.28	52.54	

Hydroxy radicals scavenging activity was assessed by generating the hydroxyl radicals using ascorbic acid-iron EDTA. The hydroxy radicals formed by the oxidation, reacts with dimethyl sulfoxide to yield formaldehyde, and provides a convenient method for the detection of hydroxyl radicals by treatment with Nash reagent. Hence, this study affirms the *in vitro* antioxidant potential of methanolic leaf extract of *M. dubia*, with results comparable higher to those of the standard compounds such as Quercetin and Vitamin C.

Evaluation of Anti-microbial activity of plant extracts

When the standard test compound Ciproflaxacin was used in the media, the plate containing *S. mutans* showed higher zone of inhibition with a value of 25.00±0.0mm, its growth being highly inhibited, when compared to the plate containing *E. coli* with a value of 23.00±0.0mm showing lesser level of inhibition (Table 6 and Figure 1). When the bacteria were treated with the leaf extracts MDL1 and MDL2, the growth of *E. coli* was inhibited to a greater extent when compared to *S. mutans*, deviating from the standard results. Hence, MDL1 has more anti-microbial activity than MDL2. The anti-microbial efficacy of methanol extract is attributed to its phytochemical resources unveiling them as potential biocidal efficacy.

Table 6. Inhibitory activity of test compounds against bacteria

Test compounds	Concentration µl (mg/ ml)	Zone of inhibition (mm)	
		E. coli	*S. mutans*
Ciproflaxacin	25 (0.1)	23.00 ± 0.0	25.00 ± 0.0
MDL1	25 (100)	09.00 ± 0.0	07.00 ± 0.0
MDL2	25 (100)	07.00 ± 0.0	06.00 ± 0.0

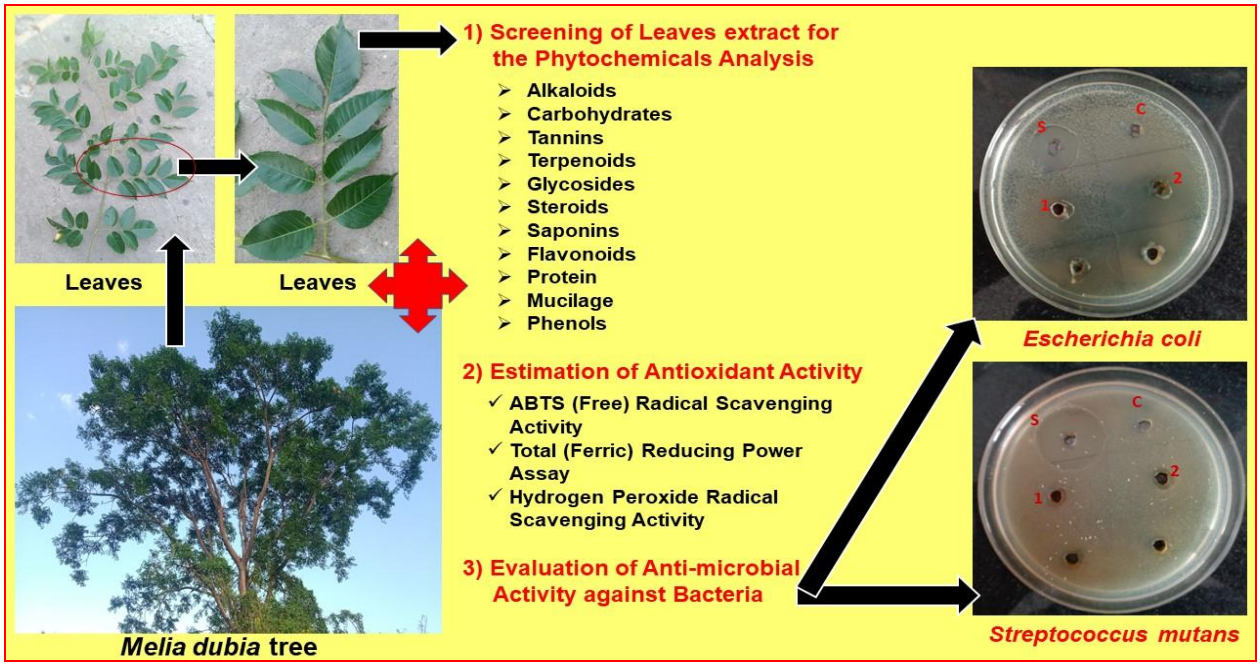

Figure 1. Inhibitory activity of test sample against bacteria and Summary of the findings. S = Standard (Ciproflaxacin), C = Control, 1 = MDL1, 2 = MDL2.

The present study claims the complete analysis of phytochemical, antioxidants and anti-microbial of *M. dubia* in the figure 1, and could be utilized to rendering health benefits also. Thus, this study demonstrates that *M. dubia* can be a potential medicinal plant for human mankind.

CONCLUSION

Phytochemical analysis of leaf extract of *M. dubia* revealed that the presence of significant secondary metabolites of plants contributes to its anti-microbial and antioxidant activity. Thus, herbal drugs obtained from the plants are relatively safe and exhibit a remarkable efficacy in the treatment. Thus, *M. dubia* can be used as a source for development of potential anti-microbial drug comparable to synthetic pesticides. Phytochemicals contents present in the leaf extracts are capable of rendering health benefits also, thereby demonstrating that *M. dubia* is a potential medicinal plant, and essentially replaced as a complement to synthetic anti-microbial agents available in market today.

ACKNOWLEDGEMENTS

We thank Dr. S. Yogesha, Director and Dr. Ananda, Technical Staff of the Skanda Life Sciences Private Limited, Sri Shaila Bramara Complex, Srigandadha Kaval, Bengaluru 560 091 for the conduct of desired experiments and providing the laboratory facilities for this work.

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