COMPARATIVE *IN-VITRO* ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF HYDROALCOHOLIC EXTRACT OF*MILIUSA TOMENTOSA*

Abstract

Green medicine, the drugs derived from plants attained a wide spread interest, as believed to be safe and dependable, compared with costly synthetic drugs that have adverse effects. The present study aimed to analyze a comparative *in vitro* free radical scavenging and antimicrobial potentials of leaf hydroalcoholic extract fractions of *Miliusatomentosa* (Roxb.) J. Sinclair (*M. tomentosa*, Annonaceae) commonly known as hoom, kari.It is a large deciduous tree, growing up to 20 m tall and are given to children to cure the weakness in summer. The hydroalcoholicextract of leaves of *M. tomentosa* was studied for antioxidant activity on different *in vitro* models namely 1,1-diphenyl, 2-picryl hydrazyl (DPPH) assay, Hydrogen peroxide (H_2O_2) and Nitric oxide (NO) radical scavenging method. Agar well diffusion method has been used to determine the antimicrobial activities of plant extracts against Gram-positive bacteria, Gram-negative bacteria (*Enterococcus faecalis, Salmonella Bongori,Propionibacterium acnes*), and fungus (*Aspergillusflavus*). In the investigated models, the extract showed dose-dependent free radical scavenging properties. For the DPPH technique, M. tomentosa leaves extract had an IC50 value of 335.92g/ml, which was close to that of ascorbic acid (IC_{50}=17.68g/ml). The IC_{50} value for hydrogen peroxide was determined to be 96.42g/ml, which compares favourably to ascorbic acid (IC50=36.61g/ml). The IC50 value for nitric oxide was 105.87g/ml, which was close to that of ascorbic acid (IC_{50}=24.63g/ml). The extracts exhibited both antibacterial and antifungal activities against tested microorganisms using standardampicilin, ciprofloxacin, clindamycin andfluconazole (10-30µg/ml). The antimicrobial activity was determined by measuring the diameter of the zone of inhibition in term of millimeter (mm). The antimicrobial activity of hydroalcoholic extract of leaves against all microorganisms was concentration dependent but less than standard drug. On the basis of results obtained, it is suggested that both *M. tomentosa* leaves extracts may be a potential source of natural antioxidants and antimicrobial compounds to be used in the treatment of various oxidative disorders, infectious diseases caused by resistant microorganisms.

Keywords: *Miliusa tomentosa,* Annonaceae, Antioxidant activity, Antimicrobial activity.

INTRODUCTION

The study of diseases and their treatment have been existing since the dawn of human civilization. Also for the treatment of a range of diseases, herbal drugs have been used since ancient times as medicines. Medicinal plants have played a key role in world health. The

World Health Organisation (WHO) estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs [12]. In spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care. A large proportion of the India population for their physical and psychological health needs depend upon traditional systems of medicine. Medicinal plants have become the focus of intense study in terms of conservation and as to whether their traditional uses are supported by actual pharmacological effects or merely based on folklore [1-3]. Recently, there is great attention towards natural antioxidants from plants. Antioxidants can act as a radical scavenger, promote health, and produce anticancer activity [4].There is a growing concern about antimicrobial resistance issue [5]. The complications of multidrug resistance enforced scientists to search for new antimicrobial agents from various sources such as medicinal plants [6].The genus *Miliusa* (Annonaceae) consists about 40 species which grows in tropical rainforest of India, Thailand, South China and North Australia [7].The different species of *Miliusa* are invariably small to large trees and are found in a wide range of rainforest communities. Only three species of Genus *Miliusa* occur in Australia, which are endemic to that contain two essential oils [8]. The plant is used in folk medicine for different symptom such as gastropathy and glomerulo nephropathy [9]. In Chinese traditional medicine *M. tomentosa* oil has been found to have both antibacterial and analgesic properties [10]. Knowledge of the chemical constituents of plants is desirable because such information will be valuable for synthesis of complex chemical substances [11]. Two new isoquinoline alkaloids, 2,10- dimethoxy-3,11-dihydroxy-5,6-dihydroprotober -berine and 1,9-dihydroxy-2,11 -dimethoxy-4,5- dihydro-7-oxoaporphine, together with thirteen known alkaloids, were isolated from the ethanolic extracts of the stem and leaves of *M. cuneata* (Graib) [12]. Since *M. tomentosa* is one of them, its traditional uses are not reported but its fruits are eaten in some parts of India and its tree yields a pale yellow gum known as karee gum [13]. Literature reviews pointed out that no studies combining the antioxidant and antimicrobial activities of the leaves of *M. tomentosa* have so far been undertaken. In this work, we wanted to evaluate the antioxidant and antibacterial activity of hydroalcoholic extracts of *M. tomentosa* leaves, which is in line with our ongoing interest in pharmacological screening of Indian medicinal plants.

MATERIALS AND METHODS

Plant material

Leaves of *M. tomentosa* were collected from rural area in month of December 2019. The leaves plant sample were separated and washed with sterile distilled water to remove the

adhering dust particles and other unwanted materials. The leaf was air dried under room temperature. The dried plant samples were cut and grinded to make it in powder form.The powdered samples were stored in clean, dry and sterile container for further use.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade. The pathogenic bacteria and fungus used in the current study obtained from Microbial Culture collection, National Centre forcell science, Pune, Maharashtra, India.

Defatting of plant material

Powdered leaves of *M. tomentosa* were shade dried at room temperature. The shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether using maceration method. The extraction was continued till the defatting of the material had been completed.

Extraction by soxhletion method

100 gram of powdered leaves of *M. tomentosa* was exhaustively extracted with hydroalcoholic solvent (ethanol-water 80:20) by soxhletion method. The extract was evaporated above their boiling points. Finally, the percentage yields were calculated of the dried extracts [14].

Antioxidant activity

DPPH radical scavenging assay

DPPH scavenging activity was measured by modified method of Olufunmiso *et al.*, 2011 [15]. DPPH scavenging activity was measured by the spectrophotometer. Stock solution (6 mg in 100ml methanol) was prepared such that 1.5 ml of it in 1.5 ml of methanol gave an initial absorbance. Decrease in the absorbance in presence of sample extract at different concentration (10-100 µg/ml) was noted after 15 minutes. 1.5 ml of DPPH solution was taken and volume made till 3 ml with methanol, absorbance was taken immediately at 517 nm for control reading. 1.5 ml of DPPH and 1.5 ml of the test sample of different concentration were put in a series of volumetric flasks and final volume was adjusted to 3 ml with methanol. Three test samples were taken and each processed similarly. Finally the mean was taken. Absorbance at zero time was taken for each concentration. Final decrease in absorbance was noted of DPPH with the sample at different concentration after 15 minutes at 517 nm. The

percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%. Though the activity is expressed as 50% inhibitory concentration (IC_{50}), IC_{50} was calculated based on the percentage of DPPH radicals scavenged. The lower the IC50 value, the higher is the antioxidant activity.

Nitric oxide (NO^o) radical scavenging assay

The determination of NO^o radical scavenging ability of the extracts is based on the inhibition of NO^oradical generated from sodium nitroprusside in phosphate buffer saline solution by Griess reagent (1% sulfanilamide, 2% orthophosphoric acid and 0.1% naphthylethylenediaminedihydrochloride). Scavengers of nitric oxide act against oxygen, prompting to lessened production of nitrite ions which can be monitored at 546 nm [16,17]. Briefly, sodium nitroprusside (0.6 ml, 5 mM) solution was mixed with and without varying the concentration of the extracts or Ascorbic acid (2 ml, 10-200µg/ml) and incubated at 25 ± 2°C for 5 h. Incubated solution (2 ml) was mixed with equal volume of Griess reagent and absorbance of the purple colored azo dye chromophore was measured at λmax546 nm using UV-Vis spectrophotometer. The NO^o radical scavenging ability was calculated using following formula:

$$\text{Scavenging activity (\%)} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} x100$$

Free *radical scavenging activity (FRSA) using hydrogen peroxide*

Scavenging activity of hydrogen peroxide (H_2O_2) by the plant extract was determined by the method of Ruch*et al.*, (1989) [18]. Ethanolic extract (4 ml) prepared in distilled water at various concentration was mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm. Ascorbic acid was used as a positive control compound. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples using following equation [19].

$$S\% = [(Acontrol - Asample)/Acontrol] \times 100$$

Where Acontrol = absorbance of the blank control (containing all reagents except the extract solution) Asample = absorbance of the test sample.

Antimicrobial activity

Method of preparation

This agar medium was dissolved in distilled water and boiled in conical flask of sufficient capacity 20 ml. Dry ingredients are transferred to flask containing required quantity (15 ml) of distilled water and heat (40^0C) to dissolve the medium completely.

Sterilization culture media

The flask containing medium was cotton plugged and was placed in autoclave for sterilization at 15 lbs /inch^2 (121^oC) for 15 minutes.

Preparation of plates

After sterilization, the media in flask was immediately poured (20 ml/ plate) into sterile Petri dishes on plane surface. The poured plates were left at room temperature to solidify and incubate at 37^oC overnight to check the sterility of plates. The plates were dried at 50^oC for *30 minutes before use.*[10]

Well diffusion method

The well diffusion method was used to determine the antimicrobial activity of the extract prepared from leaves of *M. tomentosa* using standard procedure [20]. There were 3 concentration used which are 25, 50 and 100 mg/ml for each extracted phytochemicals in antibiogram studies. It's essential feature is the placing of wells with the antibiotics on the surfaces of agar immediately after inoculation with the organism tested. Undiluted over night broth cultures should never be used as an inoculums. The plates were incubated at 37^oC for 24 hr. and then examined for clear zones of inhibition around the wells impregnated with particular concentration of drug.

Results and discussions

The crude extracts so obtained after each of the successive soxhletion extraction process were concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The yield of hydroalcoholic extracts obtained from the leaves of *M. tomentosa* was found to be 9.14%w/w. DPPH radical scavenging assay measured hydrogen donating nature of extracts [21]. Under DPPH radical scavenging activity the inhibitory concentration 50% (IC_{50}) value of *M. tomentosa* hydroalcoholic leaves extract was found to be 335.92µg/ml as compared to that of ascorbic acid (17.68µg/ml). A dose dependent activity with respect to concentration was observed Table 1 & Fig. 1. Extracts showed NO^o scavenging effects by competing with oxygen to react with NO directly hence inhibited the nitrite ion formation [22]. *M. tomentosa* hydroalcoholic leaves extract showed nitric oxide (NO^o) radical scavenging activity with IC_{50} value of 105.87µg/ml as compared to that of ascorbic acid (IC_{50}24.63µg/ml) Table 2& Fig. 2. Hydrogen peroxide is generated in vivo by several oxidase enzymes and by activated phagocytes and it is known to play an important role in the killing

of several bacterial and fungal strains [23].There is increasing evidence that, hydrogen peroxide, either directly or indirectly via its reduction product, OH^o, can act as a messenger molecule in the synthesis and activation of several inflammatory mediators [24].When a scavenger is incubated with H_2O_2 using a peroxidase assay system, the loss of H_2O_2 can be measured. Table 3& Fig. 3 show the scavenging ability of *M. tomentosa* hydroalcoholic leaves extract and ascorbic acid on hydrogen peroxide at different concentrations. Extracts was capable of scavenging hydrogen peroxide in an amount dependent manner at all the tested concentrations. Hydrogen peroxide itself is a rather weak oxidant and most organic compounds (except for some sulfur containing molecules) are virtually inert to attack by it at ordinary environmental or cellular concentrations and temperatures. In the presence of reduced transition metal ions, however, hydrogen peroxide is converted to the much more reactive oxidant, hydroxyl radical in the cells by Fenton reaction. Besides this, studies have shown that other transition metals such as copper (I), cobalt (II) and nickel (II) also take part in the process [23].Thus, the removing is very important for antioxidant defense in cell or food systems. The antibacterial and antifungal activities were determined using the well diffusion method. This method is highly effective for rapidly growing microorganisms, and the activities of the test extracts are expressed by measuring the diameter of the zone of inhibition. The antimicrobial activity of hydroalcoholic leaves extract of *M. tomentosa*showed bioactivity by inhibiting growth of microbial species selected for the test as shown in Table 4 and 5. The zone of inhibition shown by the extracts was comparable to the standard drug. It is effective against *Enterococcus faecalis, Salmonella Bongori,* and *Propionibacterium acnes* in concentration dependent manner but hydroalcoholic extract shown no activity against *Aspergillusflavus*.

Table 1: % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using DPPH method

S. No.	Concentration (µg/ml)	% Inhibition	
		Ascorbic acid	Hydroalcoholic extract
1	10	44.65	3.00
2	20	48.62	4.20
3	40	65.34	7.80
4	60	69.65	12.31
5	80	77.41	13.81
6	100	84.13	15.01
IC 50		67.68	135.92

Table 2: % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using NO method

S. No.	Concentration	% Inhibition

	(µg/ml)	Ascorbic acid	Hydroalcoholic extract
1	20	47.70	22.57
2	40	52.92	31.46
3	60	67.43	36.55
4	80	68.89	42.88
5	100	74.42	47.22
IC 50		44.63	95.87

Table 3: % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using H_2O_2 Method

S. No.	Concentration (µg/ml)	% Inhibition	
		Ascorbic acid	Hydroalcoholic extract
1	20	42.87	24.16
2	40	52.19	30.22
3	60	60.41	34.97
4	80	65.78	45.61
5	100	68.75	51.55
IC 50		36.61	96.42

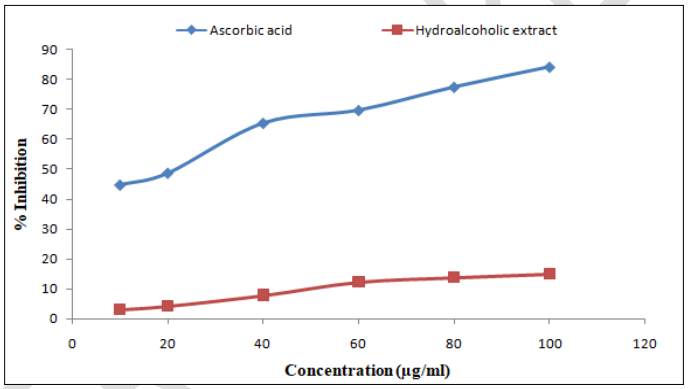

Figure 1: % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using DPPH method

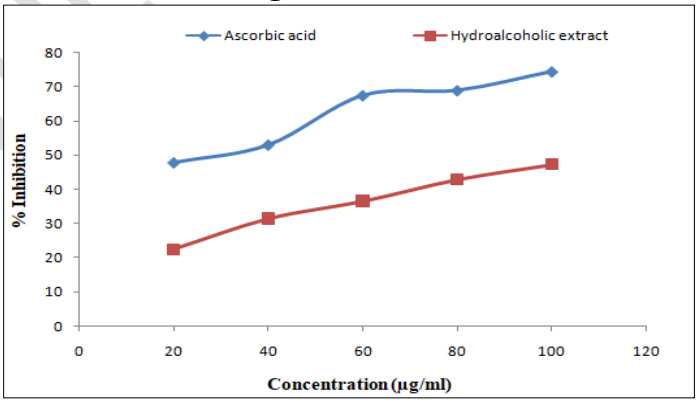

Figure 2: % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using NO method

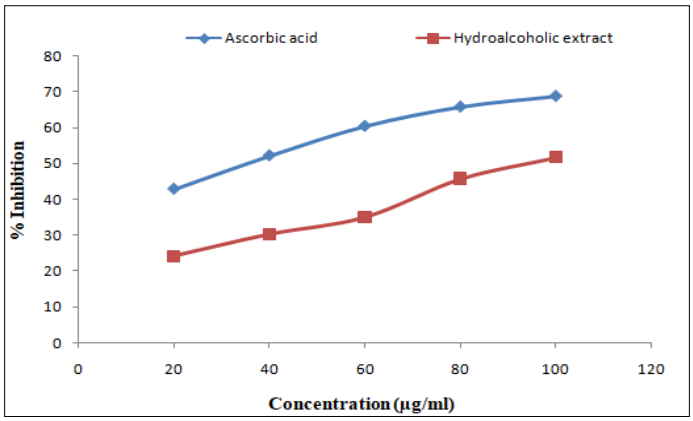

Figure 3: % Inhibition of ascorbic acid and hydroalcoholic extract of *M. tomentosa* using H_20_2 method

Table 4: Antimicrobial activity of *standard drug*against selected microbes

S.No.	Name of drug	Microbes	Zone of Inhibition		
			10 µg/ml	20 µg/ml	30 µg/ml
1.	Ampicilin	*Enterococcus faecalis*	12±0.28	15±0.57	18±0.28
2.	Ciprofloxacin	*Salmonella Bongori*	17±0.15	23±0.86	25±0.5
3.	Clindamycin	*Propionibacterium acnes*	13±0.28	16±0.57	19±0.86
4.	Fluconazole	*Aspergillusflavus*	17±0.81	24±2.94	30±1.63

Table 5: Antimicrobial activity of hydroalcoholic extract of leaves of *M. tomentosa* against selected microbes

S. No.	Name of microbes	Zone of inhibition (mm)		
		Hydroalcoholic extract		
		25mg/ml	50 mg/ml	100mg/ml
1.	*Enterococcus faecalis*	6±0.47	7±0.81	8±0.47
2.	*Salmonella Bongori*	6±0	7±0	8±0.47
3.	*Propionibacterium acnes*	8±0.47	9±0.47	10±0.47
4.	*Aspergillus flavus*	-	-	-

CONCLUSION

From the experiment it has shown that hydroalcoholic extracts have been used *in vitro* to inhibit the growth of some disease causing bacteria. It can therefore be suggested that plant extracts have great potential as antimicrobial compounds against microorganisms and they can be used in the treatment of infectious diseases caused by resistant microorganisms. They can also be a source of natural antioxidants agents. Due to their antibacterial and antioxidant activities *M. tomentosa* extracts have promising potential as a source of natural antioxidant and antimicrobial agents. Therefore these results are encouraging enough to pursue characterization of these fractions in different other models in detail. Further studies may also be conducted to isolate & purify the active constituents to evaluate others activity.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

1. Anonymous, Wealth of India, Edn 2, (5), C.S.I.R., New Delhi; 1985:27-29.
2. Naik JB, Jadge DR. Antiinflammatory Activity of ethanolic and aqueous extracts of *Carallumaadscendens.* Journal of Pharmacy Research 2009; 2(7):1228-1229.
3. IsratJahan Bulbul, Abu Hasanat Md. Zulfiker, Kaiser Hamid, Mst. HajeraKhatun, and Yesmin Begum. Comparative study of *in vitro* antioxidant, antibacterial and cytotoxic activity of two bangladeshi medicinal plants- *Luffacylindrica*L. and *Luffaacutangula.*Pharmacognosy Journal 2011, 3(23), 59-66.
4. SajidZI, AnwarF, ShabirG, RasulG, AlkharfyKM, GilaniAH."Antioxidant, antimicrobial properties and phenolics of different solvent extracts from bark, leaves and seeds of Pongamiapinnata (L.) Pierre," Molecules, 2012; 17: 3917–3932.
5. WHO, Antimicrobial resistance, Fact sheet N0 194, 2017,
6. GulluceM, Sokmen M, Daferera D. et al., "In vitro antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of Saturejahortensis L," Journal of Agricultural and Food Chemistry, 2003; 51: 3958–3965.
7. SawasdeeKT, ChaowasKU,LikhitwitayawuidK. "New Neolignans and a Phenylpropanoid Glycoside from twigs of *Miliusamollis*". Molecules, 2010; 15: 639-648.
8. JessupLW. "The genus *MiliusaLeschen*, ex A. DC. (Annonaceae) in Australia" Austrobaileya, 1988; 2: 517-523.
9. KamperdickC, VanNH, SungTV. "Constituents from *Miliusabalansae*(Annonaceae)" Phytochemistry, 2002; 61: 991-994.
10. Huong DT, VanNTH, KamperdickC, AnhNTH, SungTV." Two New Bis-styryl Compounds from *Miliusabalansae*" ChemInform, 2008; 10: 1002.

11. Mojab F, Kamalinejad M, Ghaderi N,Vanidipour HR. “Phytochemicals screening of some species of Iranian plants” Iran Journal of Pharmacy and Research, 2003; 3: 77-82.
12. JessupLW. “The genus *MiliusaLeschen*, ex A. DC. (Annonaceae) in Australia” Austrobaileya, 1988; 2: 517-523.
13. Anonymous, “The Wealth of India (Raw material) Publication of Information Directorate” Council of Scientific and Industrial Research New Delhi, 1991: 377-8.
14. Mukherjee PK. Quality Control of Herbal Drugs, 2nd Edition, Business Horizons, 2007; 2-14.
15. Olufunmiso OO, Afolayan AJ, Phenolic content and antioxidant property of the bark extract of Ziziphusmucronatawilld. Subsp. mucronatawilld, BMC Complement Alter Med. 2011; 11:130.
16. Shirwaikarkar A, Somashekar AP. Anti-inflammatory and free radical scavenging studies of *Aristyolochia bracteolate* Lam. India J Pharm Sci 2003;65:67-9.
17. Czochra MP, Widensk AJ. Spectrophotometric determination of H_2O_2 activity. Anal ChemActa. 2002; 452:177–84.
18. Ruch RJ, Cheng SJ, Klaunig JF. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis. 1989; 10: 1003–1008.
19. Marcocci L, Maguire JJ, Droy MT. The nitric oxide scavenging properties of *Gingobiloba* extract EGb 761. BiochemBiophys Res Commun. 1994; 15:748–755.
20. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology, 1966, 45:493–496.
21. Hudson BJ. Food antioxidants. In: Gordon MH, editor. The Mechanism of Antioxidant Action *in Vitro*. London: Elsevier Applied Science; 1990.
22. Sunil C, Ignacimuthu S. *In vitro* and *in vivo* antioxidant activity of *Symplocoscochinchinensis*S. Moore leaves containing phenolic compounds. Food ChemToxicol 2011;49:1604-9.
23. Halliwell B, Aeschbach R, Lolliger J, Aruoma OI, Food Chem. Toxicol. 1995, 33, 601.
24. Sprong RCA, Winkelhuyzen-Jansen C, Aarsman J, Van Oirschot T, Van deer Bruggen, Van Asbeck BJ. Am. J. Crit. Care Med. 1998, 157, 1283-1293.