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PHYTOCHEMICAL SCREENING, ANTIOXIDANT AND ANTIMICROBIAL POTENTIAL OF LEAVES EXTRACT OF *MUNTINGIA CALABURA*

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ABSTRACT

Medicinal plants have bioactive compounds which are used for cure of various human diseases and also play an important role in healing. *Muntingia calabura* L. (*M. calabura*, Elaeocarpaceae) has been traditionally used to relieve various pain-related ailments. The aim of the present study was to examine leaves of *M. calabura* for phytochemical profile. The dried leaves of *M. calabura* were successively extracted with n-hexane, ethyl acetate, methanol solvents by soxhletion method and solvents from extracts was evaporated under vacuum. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenolic, flavonoids and alkaloid content were determined by the well-known test protocol available in the literature. The *in vitro* antioxidant activity of ethyl acetate and methanolic extract of the leaves was assessed against DPPH, NO, H_2O_2 assay method using standard protocols. The preliminary phytochemical investigation revealed the presence of various phytoconstituents in each extract. To analyze the antimicrobial activity, ethyl acetate extract of *M. calabura* was tested by a well diffusion method against three selected strains which showed significant inhibitory action against all the tested strains. The present study concluded that the crude extract of *M. calabura* is a rich source of secondary phytoconstituents which impart significant antioxidant potential. The findings of the present study will be helpful to phytochemists, pharmacologists and pharmaceutical industries.

Keywords: Medicinal plants, *Muntingia calabura,* Phytochemical, Antioxidant activity.

1. INTRODUCTION

Medicinal plants are sources of important therapeutic aid for alleviating human ailments. Approximately 80% of the people in the developing countries all over the world depend on the traditional medicine for their primary health-care. Interestingly, approximately 85% of traditional medicine involves the use of plant extracts. Interest in phytomedicine has been started in the last 20 years and with increasing awareness of the health hazards and toxicities associated with unsystematic use of synthetic drugs and antibiotics, interest in the use of plants and plant-based drugs has revived throughout the world. However, a large number of medicinal plants remain to be investigated for their possible pharmacological value [1]. Such plants should be investigated to better understand their properties, safety and efficacy. The medicinal properties of plants are due to some chemical constituents that produce certain pharma-cological action on the humans. The qualitative analysis of phytochemicals of a medicinal plant is reported as vital step in any kind of medicinal plant research. Screening of plants constituents accurately can be done by employing chromategraphic techniques [2]. Quantification usually employs the use of gravimetric and spectroscopic methods with several advanced approaches now available [3].

Oxygen is essential to many living organisms for the production of energy to fuel biological processes. However, the metabolism of oxygen generates 'free radicals' which induce oxidative damage to biomacro-molecules, including DNA, proteins, membrane lipids and carbohydrates [4]. A common theme that underlies the aetiology of several degenerative disorders is free radical stress [5]. Free radicals are reported to be involved in the occurrence of numerous diseases such as cancer, diabetes mellitus, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases [6-10]. Antioxidants are vital substances because they can protect the body from the damage caused by free radicals. They exert their effect by scavenging the free radicals (*i.e.*

reactive oxygen species (ROS) or reactive nitrogen species) universally present in biological systems [10]. There is increasing interests in the natural antioxidants (*e.g.* polyphenols (flavonoids and tannins)) present in plants used for medicinal and dietary purposes, which might help to prevent oxidative damage [11]. Many synthetic antioxidants *e.g.* butylated hydroxyl-anisole, are very effective. However, they possess certain side effects and are toxic to humans [12, 13]. Hence, compounds (especially those from natural sources capable of protecting against ROS-mediated damage) may have potential applications in the prevention and/or cure of certain human diseases. One of the plants that have recently gained a medicinal plant status is *M. calabura* (Elaeocarpaceae) usually known as Jamaican cherry or ceri hutan in Malaysia, is widely cultivated in warm areas of Asia, including Malaysia and tropical America. *M. calabura* plants are able to thrive in poor soil, and they are tolerant of acid and alkaline conditions as well as drought [14]. The fruit of *M. calabura* is used for tart and jam production, while the dried leaves are used to make tea [14]. The recent studies on *M. calabura* demonstrate its positive protective effect against gastric damages [15], antibacterial activity [16], antioxidant activity [14], antidiabetic activity [17], hepatoprotective activity [18], antinociceptive activity [19] and antiproliferative activity [14]. The main constituents in *M. calabura* include the following: triterpernoids (lupenone), flavonoids (5,7-dihydroxy-flavanone, 7-hydroxyflavanone, 5-hydroxy-3, 7,8-trimethoxyflavone, 50-hydroxy 7, 8, 30, 40-tetramethoxyflavan, 3,5-dihydroxy-7, 40-dimethoxy flavone), phytosterols (β-sitostenone and β-sitosterol) and chalcones derivatives (20, 4;-dihydroxydihydro-chalcone, 20, 40-dihydroxychalcone and isoliquiriti-genin) [20]. Despite several research works on the phytochemical constituents analysis and benefits of *M. calabura* leaves, little is known about the correlation between their constituents and biological activities. Based on the many ethno medicinal values of this plant, it becomes imperative to determine the active ingredients present in leaves of the plant as well as their composition, antioxidant and antimicrobial activity.

2. MATERIAL AND METHODS

2.1. Plant material

The plant material (Leaves) for the proposed study was collected from B R Nahata College of Pharmacy Mandsaur (M.P.) in month of November, 2019. Leaves selected for the study were washed thoroughly under running tap water and then were rinsed in distilled water; they were allowed to dry for some time at room temperature. Then the plant material was shade dried without any contamination for about 3 to 4 weeks. Dried plant material was grinded using electronic grinder. Powdered plant material was observed for their colour, odour, taste and texture. Dried plant material was packed in air tight container.

2.2. 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.

2.3. Extraction by Soxhletion Method

Forty Four (44) gram of powdered leaves of *M. calabura* was exhaustively extracted with different solvent (*i.e.* n-Hexane, ethyl acetate and methanol) by soxhletion method. The extract was evaporated above their boiling points. Finally, the percentage yields were calculated of the dried extracts.

2.4. Qualitative phytochemical analysis of plant extract

The *M. calabura* leaves extract obtained was subjected to the preliminary phytochemical analysis following standard methods [21, 22]. The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, alkaloids, fats or fixed oils, protein & amino acid and tannins.

2.4.1. Total phenol determination

The total phenolic content was determined using the method of Olufunmiso *et al* [23]. A volume of 2 ml of each extracts or standard was mixed with 1 ml of Folin Ciocalteau reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5 g/l) of sodium carbonate. The mixture was allowed to stand for 15 min under room temperature. The colour developed was read at 765 nm using UV/visible spectrophotometer. The total phenolic content was calculated from the standard graph of gallic acid and the results were expressed as gallic acid equivalent (mg/100mg).

2.4.2. Total flavonoids determination

The total flavonoid content was determined using the method of Olufunmiso *et al* [23]. 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/100 mg).

2.4.3. Total alkaloids content estimation

The plant extract (1 mg) was dissolved in methanol, added 1 ml of 2 N HCl and filtered. This solution was transferred to a separating funnel, 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added. The mixture was shaken with 1, 2, 3 and 4 ml chloroform by vigorous shaking and collected in a 10 ml volumetric flask and diluted to the volume with chloroform. A set of reference standard solutions of atropine (40, 60, 80, 100 and 120 µg/ml) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV/Visible spectrophotometer. The total alkaloid content was expressed as mg of AE/100 mg of extract.

2.5. Antioxidant activity

2.5.1. DPPH free radical scavenging assay

DPPH scavenging activity was measured by modified method [23]. DPPH scavenging activity was measured by the spectrophotometer. Stock solution (6 mg in 100 ml 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 concentrations (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 each of DPPH and test sample of different concentrations 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 IC_{50} value, the higher is the antioxidant activity.

2.5.2. Nitric oxide radical inhibition assay

Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction. Sodium nitroprosside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitric ions that can be estimated by using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. The reaction mixture (6m) contained sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (PBS, pH 7.4,1 ml) and extract or standard (1 ml, 10-200 µg/ml) in DMSO at various concentrations and it was incubated at 25±2°C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was removed, sulphanilic acid reagent was added (0.33% w/v, 1 ml), mixed well and allowed to stand for 5 min for completion of diazotisation. Then, 1 ml of NEDD was added, mixed and allowed to stand for 30 min in diffused light. A purple colored chromophore was formed. The absorbance was measured at 546 nm [24]. The Nitric oxide radical scavenging ability was calculated using the following equation:

Scavenging activity (%) = $[(A_0 - A_t)/ A_0] X 100$

Where A_0 is the absorbance of the control and A_t is the absorbance of the sample.

2.5.3. Hydrogen peroxide (H_2O_2) scavenging assay

Hydrogen peroxide scavenging activity of the plant extract was determined using the procedure explained by *Jayaprakasha et al.,* [25]. A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS; pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm by using the molar absorptivity of $81 M^{-1}cm^{-1}$. Different concentrations of extract (20 to 100µg/ml) in ethanol were prepared. One (1.0) ml of ethanolic standard and test were added to 2 ml of hydrogen peroxide solution in PBS. After 10 min., the absorbance was measured at 230 nm against a blank solution that contained hydrogen peroxide solution without the extract.

The percentage of H_2O_2 scavenging of the plant extract was calculated as follows:

Scavenging activity (%)= $[(A_0 - A_t)/ A_0] X 100$

Where A_0 is the absorbance of the control and A_t is the absorbance of the sample.

2.6. Antimicrobial activity

The well diffusion method was used to determine the antimicrobial activity of the extract prepared from the *M. calabura* using standard procedure of Bauer *et al.,* 1966 [26]. The drug used in standard preparation was ciprofloxacin of IP grade and fluconazole. The antimicrobial activity was performed by using 24hr culture of *Klebsiella pneumonia, Escherichia coli* and *Candida albicans.* There were 3 concentrations 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°C for 24 hr. and then examined for clear zones of inhibition around the wells impregnated with particular concentration of drug. The diameter of zone of inhibition of each wall was recorded.

3. RESULTS AND DISCUSSIONS

The crude extract so obtained after the soxhletion extraction process, extract was further concentrated on water bath evaporation the solvent completely to obtain the actual yield of extraction. The yield of *M. calabura* extracts obtained from sample using n-hexane, ethyl acetate and methanol as solvents are depicted in the Table 1. The phytochemical analysis of *M. calabura* leaves exhibited the existence of many important bioactive secondary metabolites in n-hexane, ethyl acetate and methanol extracts, such as alkaloids, phenols, flavonoids and diterpines (Table 2).

Table 1: % Yield (W/W) of *M. calabura*

S. No.	Extract	Leaves (% Yield)
1.	n- Hexane	2.97
2.	Ethyl acetate	7.53
3.	Methanol	2.03

Total phenolic compounds (TPC) was expressed as mg/100mg of gallic acid equivalent of dry extract sample using the equation obtained from the calibration curve: Y = 0.011X+0.011, R^2= 0.998, where X is the gallic acid equivalent (GAE) and Y is the absorbance. Total flavonoids content was calculated as quercetin equivalent (mg/100mg) using the equation based on the calibration curve: Y=0.032X + 0.018, R^2=0.998, where X is the quercetin equivalent (QE) and Y is the absorbance. Total alkaloid content was calculated as atropine equivalent mg/100mg using the equation based on the calibration curve: Y=0.007X+ 0.024, R^2=0.995, where X is the Atropine equivalent (AE) and Y is the absorbance (Table 3). DPPH radical scavenging assay measured hydrogen donating nature of extracts [27]. Under DPPH radical scavenging activity, the inhibitory concentration 50% (IC_{50}) value of *M. calabura* ethyl acetate and methanol extract was found to be 70.13 and 54.37µg/ml as compared to that of ascorbic acid (17.68µg/ml). A dose dependent activity with respect to concentration was observed Table 4 and Fig.1. Extracts showed $NO^{\bullet}$ scavenging effects by competing with oxygen to react with NO directly hence inhibited the nitrite ion formation [28]. *M. calabura* ethyl acetate and methanol extract showed nitric oxide ($NO^{\bullet}$) radical scavenging activity with IC_{50} value of 72.30and 66.55µg/ml, respectively, as compared to that of ascorbic acid (IC_{50} 24.63µg/ml). *M. calabura* methanolic extract showed significant activity as compared to ethyl acetate extract (Table 5 and 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 [29]. There is increasing evidence that, hydrogen peroxide, either directly or indirectly via its reduction product, $OH^{\bullet}$, can act as a messenger molecule in the synthesis and activation of several inflammatory mediators [30]. When a scavenger is incubated with H_2O_2 using a peroxidase assay system, the loss of H_2O_2 can be measured. Table 6 and Fig. 3 show the scavenging ability of *M. calabura* ethyl acetate and methanol extract and ascorbic acid on hydrogen peroxide at different concentrations. Extract 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 [29]. Thus, the removing is very important for antioxidant defense in cell or food systems. The antimicrobial activity of ethyl acetate leave extract of *M. calabura* showed bioactivity by inhibiting growth of microbial species selected for the test as shown in Table 7 and 8. The zone of inhibition shown by the extracts was comparable to the standard drug. It is effective against *Klebsiella pneumonia, Escherichia coli* and *Candida albicans* in concentration dependent manner.

Table 2: Result of phytochemical screening of *M. calabura* (Leaves)

S. No.	Constituents	n- Hexane extract	Ethyl acetate extract	Methanol extract
1.	**Alkaloids** Hager's Test	-ve	-ve	+ve
2.	**Glycosides** Legal's Test	-ve	-ve	-ve
3.	**Flavonoids** Lead acetate Test	+ve	+ve	+ve
4.	**Diterpines** Copper acetate Test	+ve	+ve	-ve
5.	**Phenol** Ferric Chloride Test	-ve	+ve	+ve
6.	**Proteins** Xanthoproteic Test	-ve	-ve	+ve
7.	**Carbohydrate** Fehling's Test	-ve	-ve	+ve
8.	**Saponins** Froth Test	-ve	-ve	+ve

Table 3: Estimation of total phenol, flavonoids and alkaloid content of *M. calabura*

S. No.	Extract	Total phenol content (mg/ 100 mg of dried extract)	Total flavonoids content (mg/ 100 mg of dried extract)	total alkaloid content (mg/ 100 mg of dried extract)
1.	n- Hexane	-	0.746	-
2.	Ethyl acetate	11.86	1.715	-
3.	Methanol	2.8	1.521	0.578

Table 4: % Inhibition of ascorbic acid and extracts of *M. calabura* using DPPH method

S. No.	Concentration (µg/ml)	% Inhibition		
		Ascorbic acid	Ethyl acetate extract	Methanol Cextract
1.	10	44.65	21.45	25.45
2.	20	48.62	29.98	35.56
3.	40	65.34	36.65	45.58
4.	60	69.65	45.56	52.23
5.	80	77.41	55.58	61.45
6.	100	84.13	62.12	69.98
	IC 50	17.68	70.13	54.37

Table 5: % Inhibition of ascorbic acid and extracts of *M. calabura* using NO method

S. No.	Concentration (µg/ml)	% Inhibition		
		Ascorbic acid	Ethyl acetate extract	Methanol extract
1.	20	47.70	36.65	26.65
2.	40	52.92	45.58	36.69
3.	60	67.43	63.32	49.98
4.	80	68.89	71.14	55.56
5.	100	74.42	75.65	65.45
	IC 50	24.63	72.30	66.55

Table 6: % Inhibition of ascorbic acid and extracts of *M. calabura* using H_2O_2 method

S. No.	Concentration (µg/ml)	% Inhibition		
		Ascorbic acid	Ethyl acetate extract	Methanol extract
1.	20	42.87	25.65	36.65
2.	40	52.19	33.34	42.23
3.	60	60.41	42.12	55.65
4.	80	65.78	49.98	62.14
5.	100	68.75	55.45	69.98
	IC 50	36.61	82.86	52.43

Table 7: 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.	Ciprofloxacin	*Klebsiella pneumoniae*	19±4.71	28±1.24	36±1.699
		Escherichia coli	16±0.86	21±0.57	28±0.5
2.	Fluconazole	*Candida albicans*	7±0.86	19±0.57	13±0.74

Table 8: Antimicrobial activity of ethyl acetate extract of *M. calabura* against selected microbes

S. No.	Name of microbes	Zone of inhibition		
		Leaves ethyl acetate extract		
		25mg/ml	50 mg/ml	100mg/ml
1.	*Klebsiella pneumoniae*	10±0.94	11±0	12±0.47
2.	*Escherichia coli*	9±0.5	10±0.57	13±0.86
3.	*Candida albicans*	6±0	6±0	8±0.74

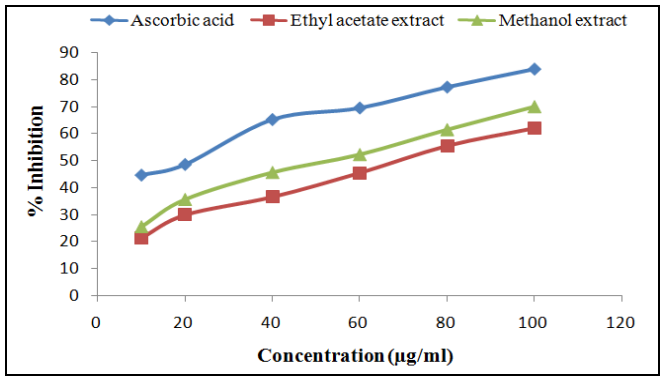

Fig. 1: % Inhibition of ascorbic acid and extracts of *M. calabura* using DPPH method

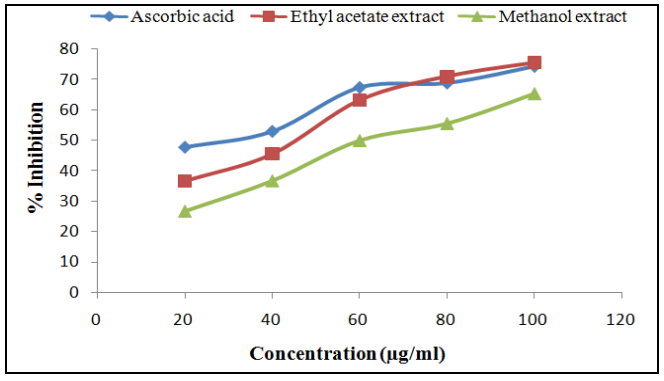

Fig. 2: % Inhibition of ascorbic acid and extracts of *M. calabura* using NO method

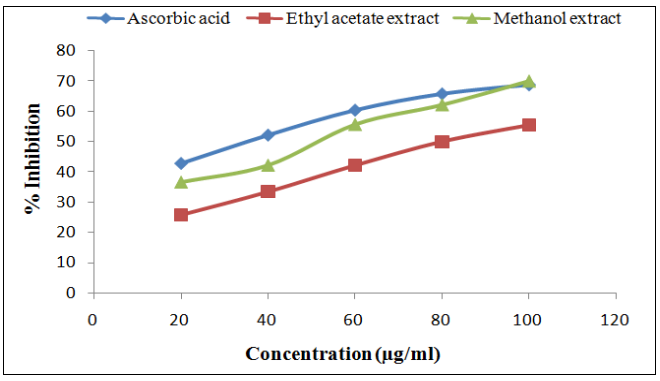

Fig. 3: % Inhibition of ascorbic acid and extracts of *M. calabura* using H_2O_2 method.

4. CONCLUSION

By considering the above data, it can be concluded that the leaves of *M. calabura* exhibited the potential antimicrobial and antioxidant activities. The leaf extracts of this plant have various phytochemicals such alkaloids, phenols, flavonoids and diterpines, which are responsible for these activities. The ethyl acetate extract has good antimicrobial activity against the tested microorganism. The plant exhibited the admirable antioxidant activity in methanol extracts. The observed good antioxidant activity of the extract indicates the potential of the leaves as a source of natural antioxidant.

Based on our findings, further studies are necessary to elucidate the mechanism lying with these effects of the plant extracts and could be open a new window in the search for new bioactive drug lead components of this plant extracts.

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