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Promising antioxidative potentiality and antibacterial activity of *Mallotus philippensis* grown in Nepal

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Abstract
Carbohydrates, glycosides, lactones, steroids, terpenoids, saponins, tannins, polyphenols, flavonoids and quinones were constituted in the bark, leaf and root of *Mallotus philippensis* that naturally grown in Nepal. Anthraquinones and anthracenosides were also found to be deposited in the bark, and the root also constituted anthraquinones. The 90% methanolic extract of the bark, leaf and root of *M. philippensis* exhibited cytotoxicity against brine-shrimp nauplii with LC_{50} values of 201.37, 794.35 and 45.50 µg/ml respectively displaying potential anticarcinogenic efficiency. The bark extract (IC_{50} = 33.54 µg/ml), leaf extract (IC_{50} = 76.22 µg/ml) and root extract (IC_{50} = 32.73 µg/ml) were found highly efficient in scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Hexane, chloroform, ethyl acetate, n-butanolic and aqueous fractions (F1-10) obtained from the bark and leaf of *M. philippensis* effectively inhibited the growth of seven human pathogens *viz. Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Serratia marcescens*, *Bacillus subtilis* and *Micrococcus luteus*.

Keywords: Antibacterial, antioxidant, brine-shrimp lethality assay, DPPH assay, phytochemical screening

Introduction
Mallotus philippensis (Lam.) Muell. Arg. (family Euphorbiaceae) is known as Kamala, Rohini, Sindhure and Kampillaka in Nepal [1]. Locally, it is used to cure diarrhoea, dysentery, bronchitis, typhoid and meningitis in districts of terai region of Nepal by Tharu community [2,3]. A few review articles have described ethenic and experimental usefulness of *M. philippensis* [4,5].
Flavonoids, steroids, terpenoids, tannins and saponins are constituted in the bark and stem of *M. philippensis* [6,7], whereas flavonoids and terpenoids are the major constituents in the leaf [8]. Bergenin, 11-O-galloylbergenin, friedelin, 3 α-hydroxy-D: A-friedoolean-3-en-2-one, 2β-hydroxy-D:A-friedooleanan-3-one, lupeol, lupeol-3-acetate, betulin, betulin-3-acetate, acetylaleuritolic acid, α-amyrin, β-sitosterol, etc. have been isolated from the stem of *M. philippensis* [9-11]. Platonoside, 4-dihydromyricetin, isovitexin, pachysandiol A, dopaol, bilariciresinol, bergenin and 4-O-galloylbergenin were isolated from the leaf by Mai *et al.* [12,13]. Recently, antibacterial activity of the extracts from the fruit [14], stem [7] and root [15] of *M. philippensis* of Indian origin has been reported. The extracts of whole plant of *M. philippensis* of Pakistani variety were used for antibacterial assay by Afzal *et al.* [16]. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity of the extracts from fruit [17] and leaf [18] of *M. philippensis* of Indian origin was recently reported. Herein, we report phytoconstituents, brine-shrimp cytotoxicity, DPPH free radical scavenging capacity of the 90% methanolic extracts of stem, leaf and root; and antibacterial activity of the different fractionated extracts of stem and leaf of *M. philippensis* grown in Nepal.

2. Materials and Methods
2.1. Plant material
Bark, leaf and root of *M. philippensis* were collected separately from Banke district, Nepal at 1,250 m above sea level. The plant was identified at National Herbarium and Plant Research Lab, Godawori, Lalitpur, Nepal. The plant materials were shade dried and ground to obtain powders.

2.2. Extraction
Each sample powder (20 g) was extracted separately using 90% methanol (200 ml × 3 days × 3 times) at room temperature. The combined extract was concentrated and dried to obtain 3.89 g (19.49%) of reddish brown solid from the bark, 3.63 g (18.14%) of green gummy extract from

the leaf and 4.51 g (22.54%) of reddish brown residue from the root powders. The plant extracts were used for phytochemical screening [19-21], brine-shrimp lethality assay [22] and DPPH free radical scavenging assay [23] following standard procedures [24].

Next, the bark and leaf of *M. philippensis* were extracted with 90% methanol followed by fractionation to obtain different fractions. The bark powder (100 g) was extracted with 90% methanol (1.5 L) at room temperature for a period of 9 days. The resultant extract was filtered and concentrated. The concentrate was successively partitioned into hexane (F1, 0.4 g), chloroform (F2, 2.1 g), ethyl acetate (F3, 4.7 g), n-butanol (F4, 10.0 g) and aqueous (F5, 9.5 g) fractions. Similarly, the leaf powder (100 g) was processed to obtain hexane (F6, 0.3 g), chloroform (F7, 1.5 g), ethyl acetate (F8, 3.0 g), n-butanol (F9, 5.1 g) and aqueous (F10, 8.6 g) fractions. Fractions F1-10 were then used for antibacterial susceptibility assay.

2.3. Brine-shrimp lethality assay

Brine-shrimp (*Artemia salina*) eggs were procured from San Francisco Bay Brand Inc., USA. Freshly hatched ten brine-shrimp nauplii were exposed to the sample in 10, 100 and 1,000 µg/ml dose levels in artificial sea water (total volume 5 ml), and then illuminated under a table lamp (60 W). After 24 hours, the number of survivors was counted. The percentage death, LC_{50} (Lethal Concentration for 50% mortality) and 95% CI (Confidence Interval) values were computed.

2.4. DPPH free radical scavenging assay

DPPH free radical solution was prepared in prior by overnight stirring of DPPH (3.9 mg, Sigma-Aldrich) in distilled methanol (100 ml) at 0°C. With proper dilution, the extract solutions were prepared with concentrations of 25, 50, 100, 250, 500 and 1,000 µg/ml in distilled methanol. To 0.5 ml of the extract solution was added 2.5 ml of the DPPH solution. A control was also prepared. These solutions were shaken and kept in the dark for 30 min. Absorbance value was measured at 517 nm using a spectrophotometer (6715 UV/Vis Spectrophotometer, JENWAY) against methanol blank. The inhibition percentage was calculated as:

Scavenging activity (% of inhibition) = $(1 - A_{sample}/A_{control}) \times 100$ where, A_{sample} and $A_{control}$ are the absorbance values of the reaction mixture with and without sample, respectively. Thus obtained data of % inhibitions were computed to calculate IC_{50} (concentration causing 50% inhibition of absorbance) values.

2.5. Antibacterial susceptibility assay

All together seven different pathogenic bacteria, Gram-negative *viz. Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (clinical), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella typhi* (ATCC 14038), *Serratia marcescens* (clinical), and Gram-positive *viz. Bacillus subtilis* (clinical) and *Micrococcus luteus* (clinical) were used as bacterial strains. Clinical bacterial strains were obtained from National Endemic Health Care Centre, Teku, Kathmandu, Nepal.

The residues of fractions F1-10 (0.05 g of each) were separately dissolved in 1 ml of dimethyl sulphoxide (concentration 50 mg/ml). The sample solution (50 µl of each) was charged into the well (6 mm) bored in bacteria stained Mueller Hinton agar plates. Amoxycillin (25 µg/disc, Oxoid Ltd, Basingstoke, Hampshire England) was used as a positive control. Zone of Inhibition (ZOI) produced was measured after incubation at 37°C for 24 hours. The experiment was conducted twice.

3. Results and Discussion

Phytochemical screening tests of the 90% methanolic extracts revealed that the bark, leaf and root of *M. philippensis* constituted carbohydrates, glycosides, lactones, steroids, terpenoids, saponins, tannins, polyphenols, flavonoids and quinones (Table 1). Additionally, bark constituted anthraquinones and anthracenosides, and root constituted anthraquinones.

Table 2 presents result of the brine-shrimp lethality assay. All the 90% methanolic extracts of bark (LC_{50} = 201.37 µg/ml), leaf (LC_{50} = 794.35 µg/ml) and root (LC_{50} = 45.50 µg/ml) displayed cytotoxic property against brine-shrimp nauplii and hence were considered pharmacologically active.

DPPH assay has been frequently used for evaluating antioxidative potentiality of a sample [25]. The 90% methanolic extracts of bark, leaf and root of *M. philippensis* exhibited a dose dependent inhibition of DPPH free radical (Figure 1). The IC_{50} value was calculated by plotting linear curves between % inhibitions *versus* concentrations using Microsoft Office Excel 2007 program, in which outlier values obtained for the later three concentrations (i.e. 250, 500 and 1,000 µg/ml) were omitted (Table 3). When compared to standard gallic acid (which showed IC_{50} = 9.54 µg/ml), the 90% methanolic extracts of bark (IC_{50} = 33.54 µg/ml), leaf (IC_{50} = 76.22 µg/ml) and root (IC_{50} = 32.73 µg/ml) of *M. philippensis* showed promising antioxidative potentiality indicating that the plant must constitutes some efficient antioxidants. Previously, Geetha *et al.* have reported the DPPH free radical scavenging capacity of the chloroform (IC_{50} = >500 µg/ml), ethyl acetate (IC_{50} = 75 µg/ml) and methanolic (IC_{50} = 12.2 µg/ml) extracts of the leaf of *M. philippensis* grown in India [18]. In their study, the extracts were obtained after successive Soxhlet extraction.

The antibacterial activity is expressed in terms of the diameter of ZOI (in mm) and the result of the antibacterial susceptibility assay of *M. philippensis* is depicted in Table 4. Among the different bark fractions (F1 to F5), n-butanolic fraction (F4) displayed the highest activity inhibiting Gram-negative bacteria *E. coli* (14 mm), *K. pneumoniae* (25 mm) and *S. marcescens* (14 mm), and Gram-positive bacteria *B. subtilis* (14 mm) and *M. luteus* (15 mm). Ethyl acetate fraction (F3) was also equally effective against *E. coli* and *K. pneumoniae*. Growth of *E. cloacae* was inhibited by fractions F1 and F2 only. The bark fractions were ineffective against *S. typhi*. Fractions of the leaf extract (F6 to F10) were comparatively more effective than the corresponding bark fractions and could inhibit the growth of all tested bacteria with ZOI range of 9-25 mm, except F8, which could not inhibit the growth of *B. subtilis.* Inhibition of the growth of Gram positive bacteria by both the bark and leaf fractions is noteworthy.

Velanganni *et al*. have reported antibacterial activity of the hexane, chloroform and ethanolic extracts of the stem of *M. philippensis* grown in India [7]. When the extracts were loaded at 7.5 mg/well dose, all the extracts were found ineffective against *E. coli*, *S. typhi* and *B. subtilis*, except the ethanolic extract was found effective against *E. coli* (ZOI 13 mm). In contrast, the bark fractions (F1 to F5) of *M. philippensis* grown in Nepal were found effective against both *E. coli* and *B. subtilis*, and *K. pneumoniae* and *M. luteus* as well at dose 2.5 mg/well; however, no significant ZOI was observed using strain *S. typhi* in this study. On the other hand, chloroform, ethyl acetate and n-butanolic fractions of *M. philippensis* (whole plant) grown in Pakistan showed effectiveness against *B. subtilis*, *Staphylococcus aureus*, *Staphylococcus*

pneumonia, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *S. typhi*, however; hexane fraction was ineffective [16]. The present study showed that the fractions of the leaf extract (F6-10) were effective against all seven tested bacteria and the hexane fraction (F6) was also found effective against *B. subtilis* and *S. typhi*. These results apparently showed the difference in quality of the plant material with respect to different geographical origin.

Table 1. Phytochemical screening of *M. philippensis*

Constituent	Name of test or reagent used	Bark	Leaf	Root
Alkaloids	(a) Mayer test	–	–	–
	(b) Wagner test	–	–	–
	(c) Draggendroff test	–	–	–
Carbohydrates	(a) Fehling Test	+	+	+
	(b) Molisch test	+	+	+
Glycosides	(a) Alkaline reagent test	+	+	+
	(b) Fehling test	+	+	+
	(c) Legal test	+	+	+
Lactones	(a) Kedde Test	+	+	+
	(b) Baljel test	+	+	+
Proteins and amino acids	(a) Xanthoproteic test	–	–	–
	(b) Biuret test	–	–	–
Fatty acids	Spot test	–	–	–
Steroids	Salkowski test	+	+	+
Terpenoids	(a) Salkowski test (terpenoids)	+	+	+
	(b) Tschugajen test (triperpenoids)	–	+	–
Saponins	Froth test	+	+	+
Tannins and polyphenols	(a) Braymer test (phenol)	+	+	+
	(b) Lead acetate test (tannins)	+	+	+
Phlabotanins	Precipitate test	–	–	–
Flavonoids	(a) Shinoda Test	+	+	+
	(b) Shibata test	+	+	+
	(c) Lead acetate test	+	+	+
Anthocyanins	2N HCl + aq. NH_3	–	–	–
Leucoanthocyanins	Isoamyl alcohol	–	–	–
Anthocyanosides	10% HCl + Na_2CO_3	–	–	–
Coumarins	(a) Alkaline reagent test	–	–	–
	(b) Fluorescence test	–	–	–
Anthraquinones	Brontiager test	+	–	+
Anthracenosides	Conc. H_2SO_4 + aq. NH_3 in ether	+	–	–
Quinones	Conc. HCl	+	+	+
Oxalates	Glacial AcOH	–	–	–
+ Present, – Not detected				

Table 2. Brine-shrimp lethality assay of *M. philippensis*

Sample	Percentage death at 24 hours/dose			LC_{50} (µg/ml)	95% CI (µg/ml)
	10 µg/ml	100 µg/ml	1,000 µg/ml		
Bark extract	8	40	74	201.37	201.37 ± 0.65
Leaf extract	18	20	58	794.33	794.33 ± 0.65
Root extract	14	80	100	5.50	45.50 ± 0.65

Table 3. Antioxidant activity of *M. philippensis*

Sample	Equation	R^2 value	IC_{50} (µg/ml)
Bark extract	y = 0.6091x + 29.571	0.999	33.54
Leaf extract	y = 0.7006x – 3.4015	0.998	76.22
Root extract	y = 0.6303x + 29.372	0.876	32.73

Table 4. Antibacterial susceptibility assay of *M. philippensis*

Bacterial strain	Zone of Inhibition shown by different samples (in mm)[a]										
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	Amoxycillin
Escherichia coli	12	11	14	14	11	14	20	25	23	23	19
Enterobacter cloacae	11	10	–	–	–	18	18	19	16	12	38
Klebsiella pneumoniae	20	10	25	25	15	17	20	16	16	13	38
Salmonella typhi	–	–	–	–	–	21	18	17	16	13	30
Serratia marcescens	9	–	12	14	10	17	17	12	12	9	12
Bacillus subtilis	10	9	13	14	10	10	17	–	14	15	22
Micrococcus luteus	11	10	13	15	13	17	18	21	18	20	21
[a]The values are in average of two experiments. (–)-Sign indicates no significant inhibition.											

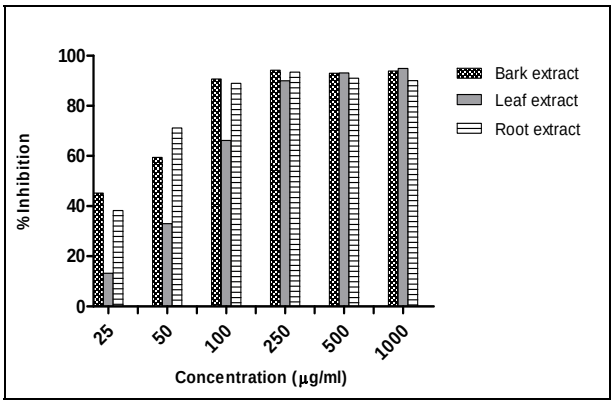

Fig 1: Percentage inhibition of DPPH free radical by the *M. philippensis* extracts

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