

**IN-VITRO ANTIOXIDANT ACTIVITY OF THE ROOTS OF
MELOTHRIA HETEROPHYLLA (LOUR.) COGN.**

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Summary

The present study investigated the preliminary phytochemical analysis and antioxidant potential in different in-vitro models of different extracts (ethyl acetate, ethanol and aqueous) of *Melothria heterophylla* Lour Cogn. root. Antioxidant activities of the extracts were evaluated using three complementary in vitro assays namely, inhibition of DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging, nitric oxide radical scavenging and superoxide anion scavenging assay and estimation of total polyphenolic content were also determined. Phytochemical screening of the plant extracts proved the presence of flavonoids, carbohydrates, gums and mucilage. All the antioxidant activities were compared with standard antioxidants such as Butylated hydroxyl toluene (BHT), ascorbic acid and curcumin. All extracts of this plant showed effective free radical scavenging activity, nitric oxide and superoxide scavenging activity. All these antioxidant properties were concentration dependent. In addition, total polyphenolic contents of all the extracts were determined as pyrocatechol (μg) equivalents. The highest antioxidant activity was observed in aqueous extract. The results obtained from the current study indicate that *M. heterophylla* Lour Cogn is a potential source of natural antioxidants.

Keywords: flavonoids, antioxidant activity, reactive oxygen species,

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Introduction

Free radicals have aroused significant interest among scientists in the past decade. Their broad ranges of effects in biological systems have drawn on the attention of many experimental works. Highly reactive free radicals, especially oxygen-derived radicals, which are formed by exogenous chemicals or endogenous metabolic processes in the human body, are capable of oxidizing biomolecules, resulting in cell death and tissue damage. Oxidative damage plays a significantly pathological role in human diseases. Cancer, emphysema, cirrhosis, atherosclerosis and arthritis have all been correlated with oxidative damage [1]. Almost all organisms are well protected against free radical damage by enzymes such as superoxide dismutase and catalase or compounds such as ascorbic acid, tocopherol and glutathione [2]. When the mechanism of antioxidant protection becomes unbalanced by factors such as ageing, deterioration of physiological functions may occur resulting in diseases and accelerating ageing. However, antioxidant supplements may be used to help the human body to reduce oxidative damage. Synthetic antioxidants are widely used because they are effective and cheaper than natural types. However, the safety and toxicity of synthetic antioxidants have been important concerns [3]. Much attention has been focused on the use of antioxidants especially natural antioxidants to inhibit lipid peroxidation or to protect the human body from the oxidative damage by free radicals. Flavonoids and other phenolic compounds (proanthocyanidins, rosmarinic acid, hydroxyl cinnamic derivatives, catechins etc.) of plant origin have been reported as scavengers and inhibitors of lipid peroxidation [4].

Melothria heterophylla (Lour.) Cogn (Family- Cucurbitaceae) popularly known as kudari is a scandent herb with tuberous roots found throughout India ascending upto 2,100 m in the hills. They are considered to be used by the tribals of Orissa for their stimulant, invigorating and purgative property [5, 6]. The juice of the leaves is applied to the parts inflamed by the application of the marking nut juice (from *Semecarpus anacardium* Linn).

The aim of the present investigation was to determine the antioxidant activity of ethyl acetate, ethanol and aqueous extract of the roots of *Melothria heterophylla* Lour Cogn.

Materials and Methods

Plant Material

The plant *Melothria heterophylla* (Lour) Cogn was identified by Botanical Survey of India, Shibpur, Howrah. A voucher specimen has been preserved in our laboratory for future reference (AD-1). After authentication, fresh root parts were collected in bulk from young matured

plants from Mayurbhanj District of North Orissa, washed, shade dried and then milled to course powder by a mechanical grinder.

Chemicals

1,1-diphenyl-2-picryl-hydrazyl (DPPH), butylated hydroxy toluene (BHT), ascorbic acid, sodium nitroprusside, nicotinamide adenine dinucleotide (NADH), nitrobluetetrazolium, phenazine methosulphate, sulphanilamide, naphthylethylene diamine dihydrochloride and potassium ferricyanide were purchased from Sigma Chemical Co. Ltd, USA. All other chemicals and reagents were analytical grade.

Preparation of Extract

The powdered plant material was extracted in succession with petroleum ether (60-80⁰C), chloroform, ethyl acetate, ethanol and distilled water using soxhlet apparatus. The solvent was then removed under reduced pressure, which gave colored residues for petroleum ether (PEMH), chloroform (CEMH), ethyl acetate (EAMH), ethanol (EEMH) and aqueous extract (AEMH) respectively. The completely dried individual extracts were used for evaluation of phytochemical constituents and in-vitro antioxidant activity.

Phytochemical screening

The extracts were screened for the presence of various constituents employing standard screening test [7]. Other conventional protocols were also used for detecting the presence of steroids, alkaloids, tannins, flavonoids, glycosides, etc.

DPPH radical scavenging effect

DPPH radical scavenging effects of the extracts were performed according to the method of Blois 1958 [8]. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of extracts solution in water at different concentrations (20, 40, 60, 80, 100 and 150 µg/ml). After 30 min, absorbance was measured at 517 nm. Butylated hydroxyl toluene (BHT) was used as a reference drug. The percentage inhibition was calculated using the formula:

$$\% \text{ inhibition} = (A_0 - A_1 / A_0) \times 100$$

A₀= absorbance of the control

A₁= absorbance of the test

Nitric oxide radical scavenging effect

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions which were measured by Griess reaction [9, 10]. The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and test extracts in different concentrations (20, 40, 60, 80, 100 and 150 µg/ml) were incubated at 25⁰C for 150 min. Each 30 min, 0.5 ml of the incubated sample was removed and 0.5 ml of Griess reagent (1 % sulphanilamide, 0.1 % naphthylethylene diamine dihydrochloride in 2 % H₃PO₄) was added. The absorbance of the chromophore formed was measured at 546 nm. Ascorbic acid was used as a standard drug. The percentage inhibition was calculated using the formula:

$$\% \text{ inhibition} = (A_0 - A_1 / A_0) \times 100$$

A₀= absorbance of the control

A₁= absorbance of the test

Superoxide anion radical scavenging effect

Superoxide anion scavenging activities of the extracts were done based on the method described by Nishimiki et al 1972 [11] with slight modification. About 1ml of nitroblue tetrazolium (NBT) solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml of NADH solution (468 µM in 100mM phosphate buffer, pH 7.4) and 0.1 ml of sample solution of test extracts (20, 40, 60, 80, 100 and 150 µg/ml) in distilled water were mixed and the reaction started by adding 100 µl of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4). The reaction mixture was incubated at 25⁰C for 5 min, and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. Curcumin was used as reference compound. The percentage of inhibition was determined by comparing the results of control and test samples. The percentage inhibition was calculated using the formula:

$$\% \text{ inhibition} = (A_0 - A_1 / A_0) \times 100$$

A₀= absorbance of the control

A₁= absorbance of the test

Determination total polyphenolic compounds

The concentrations of phenolic content in all the fractions were determined with Folin–Ciocalteu’s phenol reagent (FCR) according to the method of Slinkard and Singleton [12]. 1 ml of the sample solution (contains 1 mg) of the extract/fractions in methanol was added to 46 ml of distilled water and 1 ml of FCR, and mixed thoroughly. After 3 min, 3 ml of sodium carbonate (2%) were added to the mixture and shaken intermittently for 2 h at room temperature. The absorbance was measured at 760 nm. The concentration of phenolic compounds was calculated according to the following equation that was obtained from standard pyrocatechol graph ($R^2 = 0.9965$):

$$\text{Absorbance} = 0.001 \text{pyrocatechol } (\mu\text{g}) + 0.0033$$

Statistical Analysis

All data expressed as mean \pm SD of three parallel measurements. Data were analyzed by student’s t – test and all results were considered statistically significant at $P < 0.05$.

Results and Discussion:**Phytochemical screening:**

The respective yields after extraction of *Melothria heterophylla* root with petroleum ether (PEMH), chloroform (CEMH), ethyl acetate (EAMH), ethanol (EEMH) and aqueous extract (AEMH) and their phytoconstituents are given in table.1. The results of preliminary phytochemical screening of the five extracts revealed the presence of carbohydrates, steroids, flavonoids, saponins, gums and mucilages. Flavonoids and saponin are proved to be potent free radical scavenger [13, 14]. They are likely to act by scavenging mechanism, sacrificially reduce ROS/RNS such as $\cdot\text{OH}$, $\text{O}_2^{\cdot-}$, $\text{NO}\cdot$ or $\text{OONO}\cdot$ after generation, preventing damage to biomolecules or formation of more reactive ROS [15, 16].

Table. 1. Preliminary phytochemical constituents of the roots of *Melothria heterophylla* Lour cogn

Phytoconstituents/ % Yield	Petroleum ether	Cholorform	Ethyl acetate	Ethanol	Aqueous
Carbohydrates	+	+	-	-	-
Alkaloids	-	-	-	-	-
Glycosides	-	-	-	-	-
Proteins and Amino acids	-	-	-	-	-
Tannin	-	-	-	-	-
Terpene	-	-	-	-	-
Saponin	-	-	-	+	+
Flavonoids	-	-	+	+	+
Steroids	+	+	-	-	-
Gum and mucilage	+	+	-	-	-
% Yield	0.23	0.8	0.61	1.78	63.49

Present (+), Absent (-)

DPPH radical scavenging effect

DPPH radical scavenging activity of different extracts are given in Fig. 1. DPPH, a nitrogen centered free radical with a characteristics absorption at 517 nm and convert to 1,1-diphenyl 2- picryl hydrazine due to its hydrogen accepting ability at a rapid rate. 1,1-diphenyl-2-picrylhydrazyl (DPPH) has been used extensively as a free radical to evaluate reducing substances [17, 18, 19]. In the current study, the scavenging activities of DPPH exerted by the test samples EAMH, EEMH and AEMH as well as BHT were summarized in Figure 1. EAMH, EEMH and AEMH at the concentration of 150 µg/ml exhibited 54.64 %, 35.71% and 71.52 % inhibitions respectively, where standard drug BHT at a concentration of 100 µg/ml exhibited 90.39 % inhibition. EAMH, EEMH and AEMH shows an IC₅₀ value of 114.06 µg/ml, 220.30 µg/ml and 92.26 µg/ml respectively.

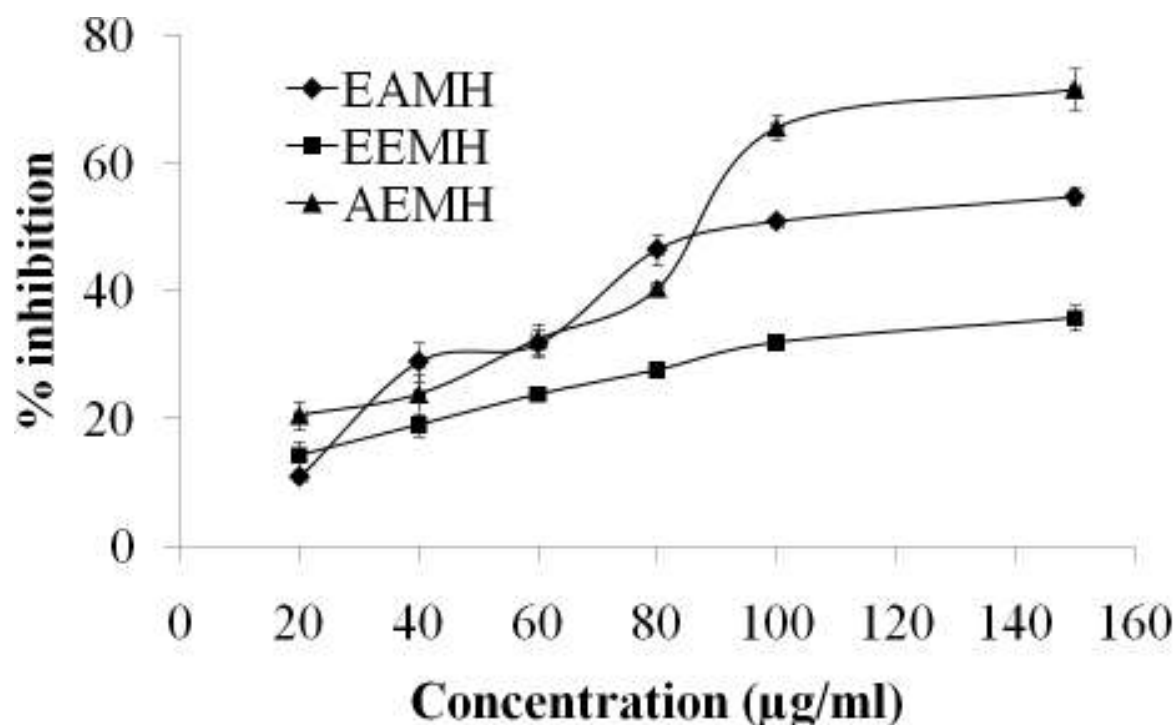


Figure 1. DPPH radical scavenging activity of the different extracts of the roots of *Melothria heterophylla* (Lour) Cogn

Nitric oxide radical scavenging effect

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and involved in the regulation of various physiological processes [20]. Excess concentration of NO is associated with several diseases [21, 22]. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals [23, 24]. In the present study, the extract competes with oxygen to react with nitric oxide and thus inhibits generation of the anions. Figure 2 illustrates the percentage inhibition of nitric oxide generation by EAMH, EEMH and AEMH. Ascorbic acid was used as a reference compound. The EAMH, EEMH and AEMH at the dose of 150 µg/ml exhibited 76.49 %, 59 % and 80.20 % inhibition respectively, whereas ascorbic acid exhibited 55.88 % inhibition at a concentration of 25 µg/ml. EAMH, EEMH and AEMH shows an IC₅₀ value of 62.43 µg/ml, 104.47 µg/ml and 50.25 µg/ml respectively.

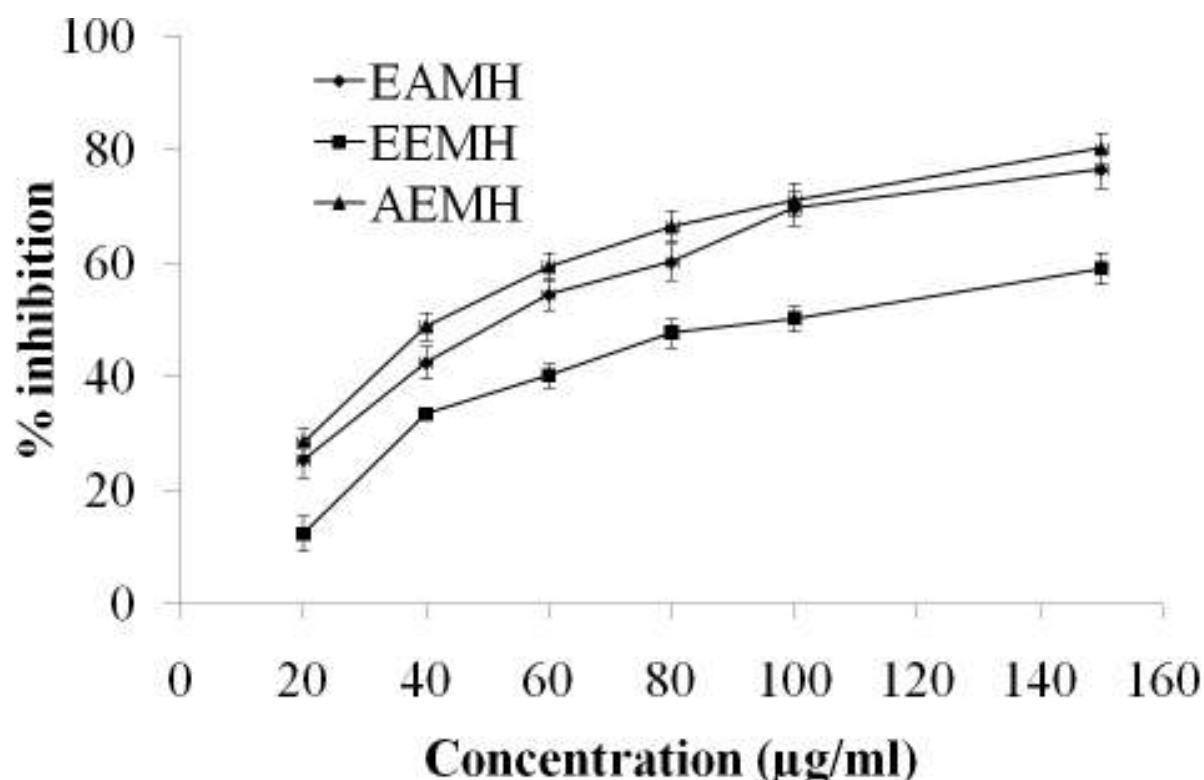


Figure 2. Nitric oxide scavenging activity of the different extracts of the roots of *Melothria heterophylla* (Lour) Cogn

Superoxide anion radical scavenging effect

In the PMS/ NADH-NBT system, superoxide radicals generated from a non enzymatic reaction of PMS in presence NADH and molecular oxygen reduces NBT to formazan at pH 7.8. Figure 3 shows the superoxide scavenging effect of EAMH, EEMH and AEMH and curcumin on the PMS/NADH-NBT system [25]. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. EAMH, EEMH and AEMH at a concentration of 150 µg/ ml inhibited the production of superoxide anion radicals by 50.61%, 32.62 % and 64.8% respectively and at the same time curcumin shows 42.21% inhibition at a concentration of 5 µg/ml. The IC₅₀ values of. EAMH, EEMH and AEMH were 126.016 µg/ml, 238.2 µg/ ml and 98.85 µg/ml respectively.

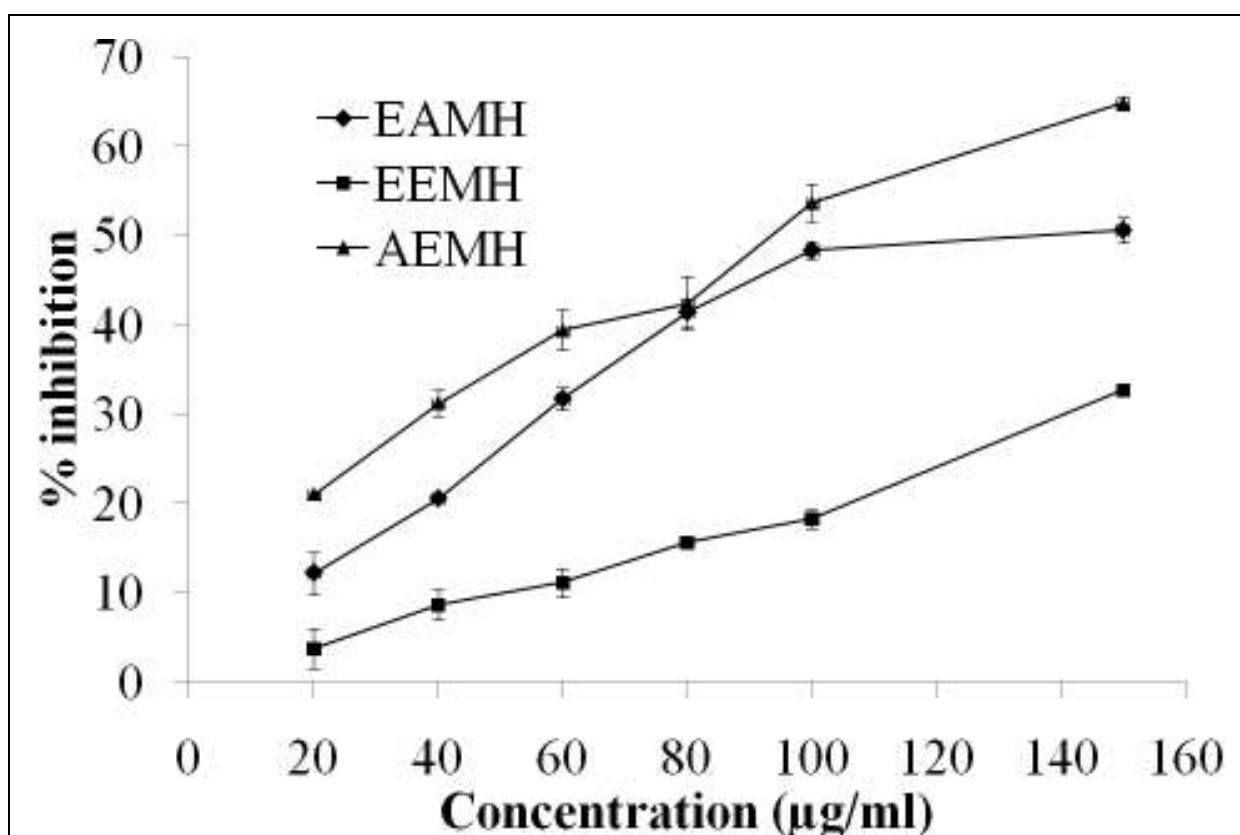


Figure 3. Superoxide anion scavenging activity of the different extracts of the roots of *Melothria heterophylla* (Lour) Cogn

Total polyphenolics contents of the extracts

Phenolic compounds are known as powerful chain breaking antioxidants. The concentration of phenolics in the extract/fraction expressed as µg of pyrocatechol per mg of the sample is shown in Table 2. Aqueous fraction was found as higher phenolics content fraction than others. The high concentration of polyphenolics in the aqueous fraction may be due to purification and concentration of phenolics throughout the fractionation procedure and probably responsible for its high free radical scavenging activity. The FCR reducing capacity of different fractions is due to presence of hydroxyl groups present in the polyphenolics and flavonoids. The key role of phenolic compounds as scavengers for free radicals is emphasized in some reports [26]. They reported that presence of hydroxyl groups contribute directly to antioxidant effect of the system and it also has an important role in stabilizing lipid oxidation.

Table. 2. Total polyphenolic content of different extracts of *Melothria heterophylla* Lour cogn.

Extracts	Phenolics as pyrocatechol equivalent ($\mu\text{g}/\text{mg}$)
EAMH	77.27 \pm 5.5
EEMH	32.45 \pm 4.05
AEMH	92.71 \pm 3.29

Further study regarding the isolation and characterization of the active principle responsible for showing antioxidant activity are currently under progress.

Conclusion

The results of the current study showed that the extracts of *Melothria heterophylla* root which contains highest concentration of flavonoids and polyphenols exhibits the greatest antioxidant activity through the scavenging of free radicals such as DPPH radical, nitric oxide and superoxide radical. So, the extracts of the roots of *Melothria heterophylla* are the source of natural antioxidants which can be accounted for the traditional uses in prevention of disease and health preservation.

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