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Varicola Karuna Sree
Assistant Professors, KVSR. Siddhartha College of Pharmaceutical Sciences, Vijayawada-10 Andhra Pradesh, India.

Meda Soundarya
KVSR. Siddhartha College of Pharmaceutical Sciences, Vijayawada-10 Andhra Pradesh, India

Maddala Ravikumar
KVSR. Siddhartha College of Pharmaceutical Sciences, Vijayawada-10 Andhra Pradesh, India.

Tiyyagura Ravichandra Reddy
KVSR. Siddhartha College of Pharmaceutical Sciences, Vijayawada-10 Andhra Pradesh, India.

Nelluri.Kanaka Durga Devi
Assistant Professors, KVSR. Siddhartha College of Pharmaceutical Sciences, Vijayawada-10 Andhra Pradesh, India.

Correspondence:
Nelluri Kanaka Durga Devi
Assistant Professors, KVSR. Siddhartha College of Pharmaceutical Sciences, Vijayawada-10 Andhra Pradesh, India.

In vitro screening of *Macrotyloma uniflorum* extracts for antioxidant and anthelmintic activities

Varicola Karuna Sree, Meda Soundarya, Maddala Ravikumar, Tiyyagura Ravichandra Reddy, Nelluri Kanaka Durga Devi

Abstract
Reactive oxygen species are generated as a result of chemical reactions taking place in the body, environmental pollution etc. Free radicals can trigger a cascade of reactions which result in damage of cell membranes, DNA, vital organs, which ultimately results in diseases like Diabetes, Alzheimer's disease, aging etc. Helminthes infestation is another major problem encountered in developing countries, where they cause more morbidity and mortality than any other infectious disease. Alcohol extracts of *Macrotyloma uniflorum* seeds were tested for their anthelmintic and antioxidant activities. These extracts exhibited potent anthelmintic activity against *Pheretima posthuma*. The activity was comparable with that of the standard, albendazole. The DPPH free radical scavenging activity of the extracts was evaluated by comparing with the standard ascorbic acid. IC50 value of the tested extracts was found to be 3.86 µg/ml. whereas the IC50 of standard was 3.95 µg/ml, which indicates a promising free radical scavenging activity. In addition to this, a sample of 100 µg/ml concentration also showed potent ferric reducing power when compared to the standard. Therefore these extracts can be used in the treatment of helminth infestations as well as free radical mediated diseases.

Keywords: *Pheretima posthuma*, Reactive oxygen species, DPPH, IC50 etc.

1. Introduction
Free radical reactions are deleterious and are the main cause for most of the degenerative diseases that afflict humanity. These diseases include Alzheimer's disease, Parkinson's disease, cataract, diabetes, rheumatoid arthritis, atherosclerosis, inflammatory joint disease, asthma, senile dementia [1], aging [2] and degenerative eye disease. Reactive oxygen species (ROS) can damage genetic material, cause lipid peroxidation in cell membranes, and inactivate membrane-bound enzymes. Humans are blessed with antioxidant defences against ROS. They include ascorbic acid (vitamin C), α-tocopherol (vitamin E), beta-carotene, coenzyme Q_{10}, enzymes such as catalase and superoxide dismutase, and trace elements including selenium and zinc. Oxidative stress can occur when there is an imbalance between antioxidant defences and production of free radicals. Oxidative damage can also cause deleterious effects on DNA leading to a number of cancers. There is an increasing demand for natural antioxidants even in food industry [3].
Helminthe infections are among the most common infections in human beings in which human intestinal parasitic worms are vectored through air, food, and water, which causes disease state, secretes toxins, and steals the vital nutrients from host bodies. The present treatment regimens for these diseases is by using anthelmintic drugs which are mainly microfilaricidal, with little effect on the adult worms; hence new drugs are urgently required. Natural products are a very good alternatives in this regard. The drugs currently used include combinations of DEC (diethylcarbamazine) and albendazole, ivermectin and albendazole or the use of DEC fortified salt. None among these is effective in killing the adult worms, which can live in the host for several years. This emphasizes the need for developing an effective and safe drug to kill or permanently sterilize the adult worms.
Macrotyloma uniflorum var benadiriamum (formerly Dolichos benadirianus chiov), commonly known by the name horse grain [4, 5], is the most extensively grown pulse in South India. It is mainly grown as feed and fodder for the cattle and especially horses. It is used as green manure.
Its important medicinal uses are, elimination of kidney stones, lowering cholesterol levels, treatment of asthma, and urinary disorder. *Macrotyloma* has the greatest potential for further

utilization as nutraceuticals forage and food for malnourished and drought prone areas of the world. Recently this plant was reported to possess cytotoxicity. Horse gram water is prescribed for treating jaundice in Andhra Pradesh [6, 7, 8].

2. Materials and methods

2.1 Plant material

Macrotyloma unifloram seeds were purchased from the nearby grocery and authenticated. The seeds were thoroughly dried and pulverised in a household mixer to obtain a coarse powder.

2.2 Chemicals

Acetic anhydride (Loba chemicals), Alkaloidal reagents (Loba chemicals), Ammonia ((Loba chemicals), Ascorbic Acid, Chloroform (Merck specialities pvt. Ltd.,), 1,1-diphenyl, 2-Picryl hydrazyl (DPPH) from Sigma Aldrich, U.S.A., Ferri chloride, Gum acacia (loba chemicals,) Hydrochloric acid (Hi-pure chemicals), Lead acetate (Hi-pure chemicals), Methanol (loba chemicals), Ninhydrin reagent (Hi-Pure chemicals), Phosphate buffer(pH6.6), Potassium ferricyanide, Pyridine (Loba chemicals), Sodium chloride (loba chemicals), Sodium hydroxide (SD fine chemicals), Sodium nitroprusside (Loba chemicals), Sulphuric acid (Hi-pure chemicals), and Trichloroacetic acid are the chemicals used.

2.3 Extraction

The coarse powder of *Macrotyloma uniflorum* is subjected to successive extraction by maceration using different solvents like chloroform, methanol, and water. The aqueous extract is obtained using soxhlet apparatus. The drug powder and the required solvent was mixed in the ratio of 1:3 and macerated. The contents are filtered by applying vacuum. The marc is re-extracted with same solvents (in 1:1 ratio), and filtered under vacuum. The combined filtrates were concentrated under vacuum and residue is stored in desiccator. The drug powder left after methanol extraction was subjected to soxhlet extraction for twelve hours, concentrated under vacuum, dried and stored in a desiccator.

2.4 Phytochemical Screening

The extracts obtained were weighed and yield was calculated. They were coded as follows:

Name of the extract	Code
Macrotyloma uniflorum chloroform extract	MUC
Macrotyloma uniflorum methanol extract	MUM
Macrotyloma uniflorum aqueous extract	MUA

The crude extracts were subjected to phytochemical investigation following standard procedures [9, 10].

2.5 Evaluation of anthelmintic activity

Indian adult earthworms (*Pheretima posthuma*) were used to study anthelmintic activity. Earthworms were collected from the water logged areas and then washed with normal saline to remove soil and fecal matter. Healthy earthworms of 5-8 cm in length and 0.2-0.3 cm in width were used in all experimental protocols [11]. Samples were prepared by dissolving 2.5 gm of crude extract in 25 ml of 1% gum acacia solution prepared in normal saline. 50 and 100 mg/ml conc. of each extract were used in the study. Standard drug, Albendazole 50 mg/ml was prepared in 1% gum acacia (prepared in normal saline solution). Along with treatment groups, a control group was also used in the study. The samples were taken in petriplates and adult healthy earth worms (n=6) were introduced into them. Observations were made for the time taken to paralyze and time taken to kill individual worms. Paralysis was said to occur when the worms do not revive even in normal saline. Death was concluded when worms lost their motility, followed by fading away their body colour [12].

Group	Extract	Code
Group 1	Methanol extract (50 mg/ml)	MUM_1
Group 2	Methanol extract (100 mg/ml)	MU M_2
Group 3	Albendazole (50 mg/ml)	S
Group 4	1% gum acacia	C

2.6 Evaluation of anti-oxidant activity

2.6.1 DPPH Radical scavenging activity

The method described by Kumaran [13] with slight modifications is used. DPPH free radical has peek absorbance at 517 nm. But, in the presence of antioxidants the colour of the reaction mixture fades and the absorbance thereby decreases. 0.1 ml of Plant extract and standard (ascorbic acid) solutions of different concentrations viz. 2.5, 5, and 10 µg/ml were added to 3 ml of a 0.004% methanolic solution of DPPH. A solution of Methanol and DPPH in equal amounts served as a control. After 30 minutes incubation in the dark, absorbance was recorded at 517 nm against blank sample. The percentage inhibiting activity was calculated from [(A0–A1)/A0]×100, where A0 is the absorbance of the control, and A1 is the absorbance of the extract/standard. Higher is the radical scavenging activity of the sample, lower is the absorbance value. The antioxidant activity of the extracts were expressed in terms of IC50. The IC50 is defined as the concentration (in µg/ml) of extracts that inhibits the formation of DPPH radicals by 50%. All the tests were performed in triplicate and the graph was plotted with the average of three observations.

2.6.2 Ferric reducing power determination

Reducing power of the methanol extracts was determined by Yen and Chen method. Different concentrations of plant extract and standard, ascorbic acid viz. 10, 20, 40, 60, 80 and 100 µg/ml in 1ml of methanol were mixed with phosphate buffer (2.5 ml, 0.2 M pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of tricholoroacetic acid (10%) was added to the mixture, to stop the reaction. The contents were then centrifuged at 3,000 g (rpm) for 10 min at room temperature. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (FeCl3) (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increase in absorbance indicates increased reducing power. Ascorbic acid was used as a positive control. All the tests were performed in triplicate and the graph was plotted with the average of three observations [14].

3. Results and discussion

%Yield was calculated using formula:

$$\% \text{ Yield} = \frac{\text{Weight of the extract}}{\text{Weight of the plant material}} \times 100$$

percentage yield of seed extracts of *Macrotyloma uniflorum* are given in Table1. Chloroform, methanol, and aqueous extracts of *Macrotyloma uniflorum* yielded 0.75%, 15.5% and 11.3% respectively. The maximum percentage of yield was reported with methanol extract, followed by aqueous extract and chloroform extract.

Table 1: Yield of Extracts

S. No	Extract	Weight in gm	%Yield
1	MUC	0.75	0.75
2	MUM	15.5	15.5
3	MUA	11.3	11.3

Phytoconstituents present in various extracts was revealed by preliminary phytochemical screening. The chloroform extract was found to contain alkaloids, carbohydrates and phytosterols. Methanol extract contains alkalolids, fixed oils and fats, carbohydrates and phytosterols. Aqueous extract was found to contain fixed oils, carbohydrates and phytosterols. Methanol extract was used in further studies. With a basic knowledge of the chemical constituents present in the crude extracts, the pharmacological activities can be predicted. The results are displayed in table 2.

Table 2: Preliminary Phytochemical Screening

Phytoconstituent	MUC	MUM	MUA
Alkaloids	+	+	-
Proteins and Amino acids	-	-	-
Fixed oils and fats	-	+	+
Flavonoids	-	-	-
Saponins	-	-	-
Carbohydrates	+	+	+
Phytosterols	+	+	+

+ indicates presence, - indicates absence

4. Evaluation of Anthelmintic activity

The results of Anthelmintic activity are shown in Table 3 and fig 1 & 2. Results are expressed as an average of six observations. Among the extracts, methanol extract (50 mg/ml) took more time to paralyse and kill the worms. At this dose, the time required to paralyze the worms was, 68±0.30 min and death was recorded at 83 ±0.20. Whereas the time required paralyzing and killing the worms with standard (Albendazole) was 45.0±0.45 and 55.0±0.20 min. respectively. The activity of 100 mg/ml methanol extracts was almost equal to that of the standard drug. The potency of the extracts was inversely proportional to the time taken for paralysis /death of the worms. The control group animals were alive up to 24 hrs. Different mechanisms by which chemotherapeutic agents act as anthelmintics are by disruption of neuromuscular physiology, blockade of energy metabolism, disrupting reproductive system [15, 16] etc. Due to time lapse the exact mechanism by which tested extracts may act as anthelmintics is not clearly known, but, it may occur by one or a combination of these mechanisms. However according to the literature, alkaloids act by interfering with energy generation in helminth parasites by uncoupling oxidative phosphorylation. Alkaloids and phytosterols present in the extracts may be responsible for the activity [17]. As the extracts caused paralysis as well as death, they can be confirmed to possess significant Anthelmintic activity. Hence these extracts can be used as anthelmintics.

Table 3: Anthelmintic activity of the samples

Group	Time of paralysis (min)	Time of death (min)
MU M_1	68±0.30	83 ±0.20
MU M_2	50±0.20	58±0.30
Albendazole	45 ± 0.45	55 ± 0.20

Fig 1: Earth worms at initial stage

Fig 2: Earth worms in final stage

4.1 Antioxidant activity

The role of free radicals in several health problems has been confirmed by reports cited earlier. Abnormally high levels of free radicals and decreased antioxidant protection are the main cause for diabetic complications, cancer, aging, obesity [18], etc. Therefore before planning for *in vivo* experiments, free radical scavenging activity and antioxidant potential of the selected fractions was investigated. The anti-oxidant activity of methanol extracts of *Macrotyloma uniflorum* seeds was evaluated by using DPPH method and Ferric reducing power determination method. The results of these assay's are depicted in table 4, 5. DPPH radical is a stable free radical, during reaction with antioxidants it is reduced which can be visualized as a change in the color from purple to yellow [19]. All the tested extracts bleached DPPH indicating their free radical scavenging activity. The standard showed a percentage inhibition of 35% at a dose of 2.5 µg/ml. whereas, the test at the same dose showed 33.14% inhibition. This indicates a promising antioxidant activity. All extracts at tested concentrations showed antioxidant activity. Further, the activity was found to be dose dependant. IC50 values for standard and methanol extracts were found to be 3.95 µg/ml and 3.86 µg/ml. Therefore these extracts possess a significant radical scavenging activity.

Reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. Therefore in Ferric reducing power determination assay, the presence of antioxidants in the samples would result in the reduction of Fe3+ to Fe2+ by donating an electron. Amount of Fe2+ complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance values at 700 nm indicates an increase in reductive ability [20]. The tested extracts showed a progressive increase in their reducing power as indicated by an increase in their absorbance values. Further, the tested extracts exhibited comparable reducing ability with the standard. The activity was dose dependant. The results of reducing power are depicted in table 6, fig 3. Methanol extracts were found to have significant DPPH free radical scavenging activity and reducing power.

These *in vitro* activities may be attributed to the phytochemical constituents present in the methanol extracts i.e. alkalolids, fixed oils and fats, carbohydrates and phytosterols whose antioxidant and free radical potentials are cited in literature [21, 22].

Table 4: Radical scavenging activity of Std.

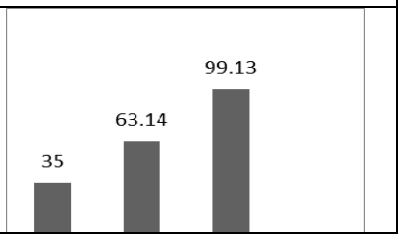
S. No	Conc. (µg/ml)	%Scavenging activity	IC50 (µg/ml)	Dose response
1	2.5	35.0	3.95	
2	5.0	63.14		
3	10	99.13		

Table 5: Radical scavenging activity of *Macrotyloma uniflorum* extract

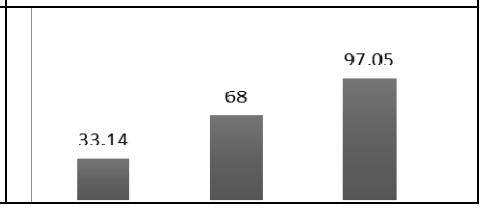
S. No	Conc (µg/ml)	%Scavenging activity	IC50(µg/ml)	Dose response
1	2.5	33.14	3.86	
2	5.0	68.0		
3	10	97.05		

Table 6: Ferric reducing power determination

S.no	Concentration (µg/ml)	Abs of MUM	Abs of Std
1	20	0.210	0.242
2	40	0.223	0.287
3	60	0.254	0.287
4	80	0.273	0.310
5	100	0.297	0.362
6	Control	0.140	0.140

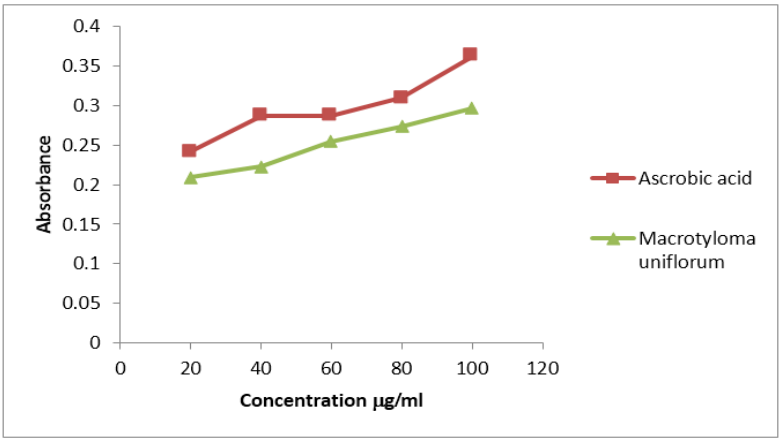


Fig 3: Reducing power of test and standard

5. Conclusion

Macrotyloma uniflorum is a legume plant which is used as food in several parts of India .Seed extracts of *Macrotyloma uniflorum* showed profound anthelmintic activity. The extracts also exhibited potent free radical scavenging activity and reducing ability. Hence the use of *Macrotyloma uniflorum* extract can be recommended as an antihelminthic, Antioxidant and free radical scavenging agent.

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