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Research Article

Antimicrobial and Cytotoxic Effects of *Macrotyloma uniflorum* Extract

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ABSTRACT

Context: *Macrotyloma uniflorum*, commonly called horse gram, is a leguminous crop. It is known to play a key role in plant defense responses with its unique characteristics. Aim: As the effect of *Macrotyloma uniflorum* extracts on the area of cytotoxicity is unexplored, the present work was aimed to evaluate the effect of extracts on cancer cell line and also on clinical pathogens following experimental and analytical procedures. Methods: Phytochemical and analytical studies (TLC and GC-MS) showed that *Macrotyloma uniflorum* is rich in different bioactive compounds. Antimicrobial activity of *Macrotyloma uniflorum* was also checked against nine clinical pathogens and methanol extract has shown moderate activity against most of the tested pathogens. Results: Moderate to high antioxidant and anticancer activity of *Macrotyloma uniflorum* extract against MG 63 cell line, has supported the fact that this crop can be used as an alternate functional food with high nutrition value.

Keywords: Horse gram, Phytochemical studies, Analytical studies, Clinical pathogens, MG 63 cell line.

INTRODUCTION

Traditionally, selection of particular diets, food practices and patterns by populations around the world was influenced by a number of factors ranging from local climatic conditions to ethno botanical and cultural practices. Furthermore, prevalence of local food items and richness of bioactive compounds generated during the course of adaptation to local stressful environmental conditions were other important criteria for their incorporation in routine diets[1]. However, in the era of globalization and modernization of food practices, the natural matrix of traditional dietary practice to which a population was exposed and adapted has changed drastically[2]. Grain legumes/pulses play an important role in the traditional diets of many parts of the world and they are low in fat; are excellent sources of protein, dietary fiber, a variety of micronutrients and phytochemicals[3,4]. *Macrotyloma uniflorum* is commonly known as horse gram (Fabaceae). It is aherbaceous plant with annual branches, sub erect or twining, leaflets 2.5-5 cm and widely distributed throughout Asia, Africa and Australia. It grows in dry zones of Southern India. Traditional medicinal texts describe its use for asthma, bronchitis, leucoderma, urinary discharges, kidney stones and heart disease[5]. It is known as poor man's pulse crop in Southern India and is a potent source of antioxidant-rich food grain[6,7]. This plant is highly resistant to disease by the pathogenic microflora. There were only few fungal diseases like root rot, anthracnose, rust and viral infections like yellow mosaic disease to mention which infect the horse gram plant. This plant shows great resistance towards the attack by normal microbial flora, which interferes with the physiological functions of the plant[8]. Although horse gram is reported to play an important role in modern dietary regimen, cytotoxic effect has not been explored much. In this study methanol and ethanol extract of *Macrotyloma uniflorum* was evaluated for antimicrobial, antioxidant and anticancer effects against osteocarcoma by experimental approaches.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents used in this study were of high purity and analytical grade.

Sample extraction

The *Macrotyloma uniflorum* were collected dried and powdered. Powdered sample were extracted with methanol and ethanol using soxhlet apparatus. The extracted solvent is later collected, dried and stored for further studies.

Phytochemical studies

Phytochemical studies were done with methanol extract of plant leaves to primarily detect the presence of various compounds.

Detection of alkaloids

Solvent free extract, 5 mg was stirred with few ml of diluted hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents[9].

A. Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids was confirmed by the formation of yellow coloured precipitate.

B. Wagner's Test: To two ml of filtrate, few drops of Wagner's reagent were added along the side of test tube. A reddish brown precipitates indicated positive test[10].

Wagner's reagent: iodine (1.27g) and potassium iodide (0.92 g) was dissolved in 5 ml of water and made up to 100 ml with distilled water.

Detection of carbohydrates and glycosides

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Table 1: Phytochemical study of Macrotyloma uniflorum methanol extract.

S. No.	Phytochemical test	Result
1	Alkaloids	
	Hager's test	+
	Wagner's test	+
2	Carbohydrates	
	Fehling's test	+
	Molish Test	+
3	Phytosterols	
	Libermann Burchard's Test	+
4	Phenols	
	Ferric chloride test	+
5	Flavonoids	
	Alkaline Reagent Test	+
	Lead acetate Test:	+
6		+
	Terpenoids	+
7	Salkowski test	+

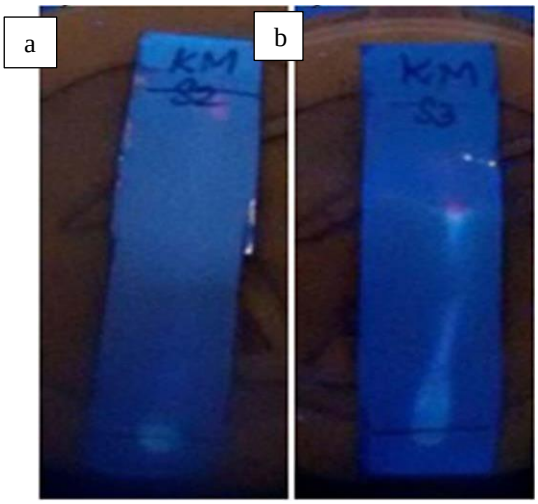


Figure 1: Separation of compounds of Macrotyloma uniflorum (a) methanol and (b) ethanol extract on TLC plate.

5 mg of extract was dissolved in 5 ml of water and filtered. The filtrate was subjected to the following tests[11].

A. Fehling's Test: 1 ml of filtrate was boiled on water bath with 1 ml of each of Fehling's solutions A and B. Appearance of red precipitate confirmed the presence of sugar.

Fehling's solution A: Copper sulphate (34.66 g) was dissolved in distilled water and made up to 500 ml using distilled water.

Fehling's solution B: Potassium sodium tartarate (173 g) and sodium hydroxide (50g) was dissolved in water and made up to 500 ml.

B. Molish Test: to 2 ml of filtrate, two drops of alcoholic solution of α napthol were added, the mixture was shaken well and 1 ml of conc. sulphuric acid was added slowly along the sides of test tube and allowed to stand. Formation of violet ring indicated the presence of carbohydrates.

Detection of phytosterols

Libermann Burchard's Test: The extract (5 mg) was dissolved in 2 ml acetic anhydride. To this, one or two drops of conc. sulphuric acid were added slowly along the sides of the test tube. An array of colour changes indicated the presence of phytosterols[12].

Detection of phenolic compounds

Ferric chloride test: The extract (2 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. Appearance of green colour indicates the presence of phenolic compounds[13].

Detection of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, which indicated the presence of flavonoids.

Lead acetate Test: one ml of the plant extract was added in a test tube. To this 1ml of 5% lead acetate and the mixture was allowed to stand for few minutes. The formation of precipitate in the sample confirmed the presence of flavonoids.

Detection of Tannins

About 0.5 g of extract was stirred with about 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate. Formation of a bluish black, bluish green or green precipitate confirmed the presence of tannins.

Detection of terpenoids (Salkowski test)

Two ml of chloroform was added to the extract. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. Appearance of reddish brown colouration on the interface indicate the presence of terpenoids.

Analytical methods

Fractionation of the crude extract using TLC

Using pre-coated TLC F254 plates, the crude extract was fractionated using different combinations of hexane/chloroform/methanol solvents (2:1:1) and methanol/hexane (3:2) as the mobile phase. Separated components were viewed in visible light, under UV at 360 nm, by fluorescence quenching less than 254 nm. Separation was done with mobile phase comprising of hexane: chloroform: methanol which was fractionated and recorded.

Gas Chromatography Mass Spectrometry

Methanol and ethanol extract of *Macrotyloma uniflorum* were analyzed by GC-MS. Perkin Elmer Clarus 680 gas chromatographic instrument equipped with a mass spectrometer detector (Clarus 600 model) and an Elite-5MS (30.0 m, 0.25 mmID, 250 µm df) column was used. The carrier gas used was helium at a flow rate of 1 ml min^{-1}. The following temperature program was used: initially the oven temperature was held at 60°C for 2 min and then ramped from 10°C/min to 300°C withhold time for 4 min, total run time 30 min. The temperature of the injector was maintained at 300°C. The ion trap was operated at 70 eV with a scan range of m/z from 50 to 600. A sample of 1 µl was injected in split mode (10:1). The intermediate and end product was identified based on the Wiley registry of mass spectral data.

Antimicrobial study

The antibacterial activity of methanol and ethanol extract of *Macrotyloma uniflorum* against nine bacterial pathogens were evaluated by using agar well diffusion

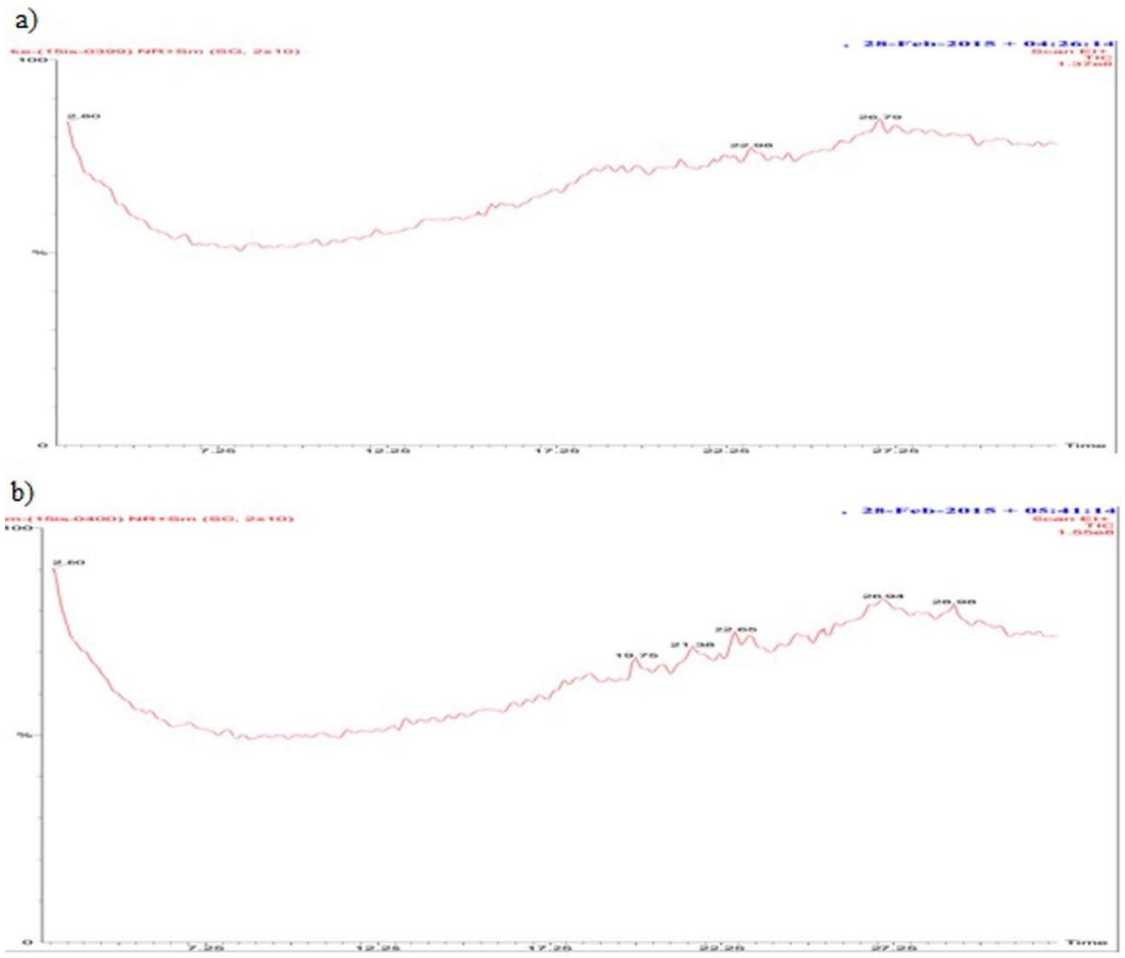


Figure 2: GC-MS chromatogram of Macrotyloma uniflorum methanol and ethanol extract.

method[14,15]. Muller Hinton Agar (MHA) plates were inoculated with selected bacterium. Wells of 8 mm size were made with sterile borer on agar plates. Four different volumes (25 µl, 50µl, 75 µl, 100 µl) of the plant extract were poured into each well of inoculated plates. Respective solvent for particular solvent extracts was used as a negative control. Then they were left at room temperature for ten minutes allowing the diffusion of the plant extract into the agar[16] and incubated in the incubator. After incubation for 24 hrs at 37°C, the plates were observed for clear zone. Antibacterial activity of the extract was identified by zone of inhibition surrounding the well containing the plant extract which was measured and expressed in millimeters (mm). Clear zone of the plant extract and comparison with negative control was also recorded[17].

Antioxidant Study

The antioxidant activity of the methanol and ethanol extract was evaluated by DPPH radical scavenging assay which was originally described by Blois[18]. DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a synthetic free radical with deep violet colour when is in the form of solution which has a λ_{max} at 517nm. It can accept an electron or hydrogen radical to become stable diamagnetic molecule and appear as light purple in colour which indicates the scavenging of DPPH and the substance has antioxidant activity.

Methanol solutions were prepared with all the three extracts. Methanol solution of DPPH was used as negative control. 500µl of each sample and 500 µl of DPPH solution was allowed to react and incubated at room temperature for 30 mins under dark conditions. Absorbance was taken at λ_{max} i.e. 517nm against a blank which was 500µl of methanol. Percentage inhibition was calculated by the following equation to conclude the presence of antioxidant activity of the extracts.

Percentage of inhibition = (OD control – OD sample / OD control) x 100

Anticancer study

Cell line

The human osteosarcoma cell line (MG 63) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. One hundred

Table 2: Antimicrobial activity of Macrotyloma uniflorum ethanol extract.

S. No.	Organisms	Zone of inhibition (cm)			
		25mg/ml	50mg/ml	75mg/ml	100mg/ml
1	Pseudomonas aeruginosa	-	-	-	-
2	Shigella sp.	-	-	-	-
3	Serretia sp.	-	-	-	-
4	Salmonella sp.	-	-	-	-
5	Klebsiella sp.	1.0	1.2	1.3	1.5
6	Enterobacter sp.	-	-	-	-
7	Proteus mirabilis	0.7	0.9	1.0	1.2
8	Staphylococcus sp.	-	-	-	-
9	Escherichia Coli	-	-	-	-

Table 3: Antimicrobial activity of Macrotyloma uniflorum methanol extract.

S. No.	Organisms	Zone of inhibition (cm)			
		25mg/ml	50mg/ml	75mg/ml	100mg/ml
1	Pseudomonas aeruginosa	0.9	1.1	1.3	1.5
2	Shigella sp.	-	-	-	-
3	Serretia sp.	1.3	1.5	1.7	2.0
4	Salmonella sp.	0.5	0.6	0.9	1.1
5	Klebsiella sp.	1.0	1.2	1.3	1.5
6	Enterobacter sp.	-	-	-	-
7	Proteus mirabilis				
8	Staphylococcus sp.	-	-	-	-
9	Escherichia Coli	0.7	0.9	1.0	1.2

microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37˚C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37˚C, 5% CO_2, 95% air and 100% relative humidity. The medium without samples served as control and triplicate was maintained for all concentrations.

MTT assay

After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37˚C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader [19, 20].

The percentage of cell viability was then calculated with respect to control as follows

Percentage of cell viability = [A] Test / [A]control x 100

The percentage of cell inhibition was determined using the following formula.

Percentage of cell inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between percentage of cell inhibition and Log concentration and IC50 was determined using GraphPad Prism software.

RESULTS

Phytochemical study

In recent years, secondary plant metabolites are being extensively investigated as a source of pharmaceutical compounds. It has been accepted that natural compounds play an important role in health care. Result of phytochemical studies of *Macrotyloma uniflorum* extract with methanol is presented in a table form in table 1, confirming the presence of carbohydrate, protein, amino acid, terpenoids, saponins, flavonoids, alkaloids, steroids, glycosides and phenols are present in *Macrotyloma uniflorum*. According to Singh et al.[21] phenolic compounds possess biological properties such as antiapoptosis, antiaging, anticarcinogen, antiinflammation, as well as inhibition of angiogenesis and cell proliferation activities. Phytosterol acts as growth hormones in plants. The plant has medicinal property due to presence of these phytochemicals[22].

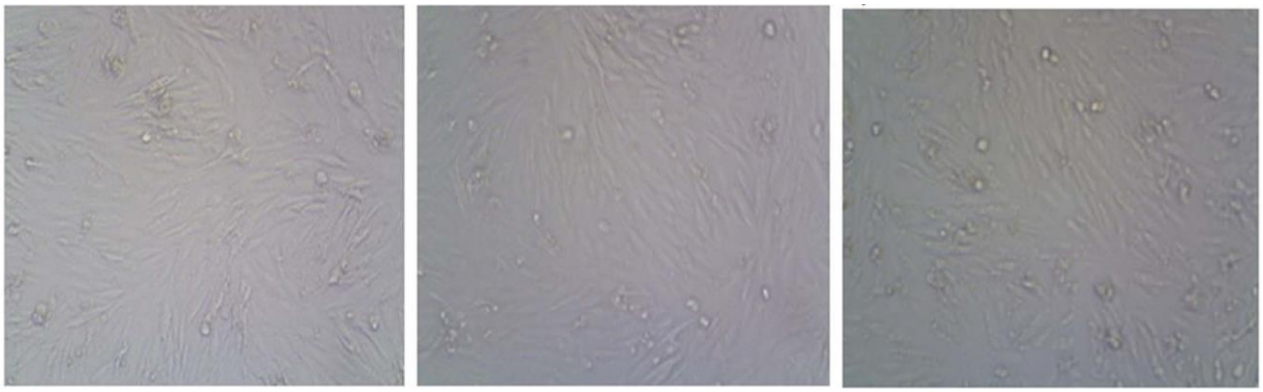
Analytical studies

TLC

TLC was done to fractionate each components of the extract by its characteristic Rf values. Separated spots were observed under UV light (fig.1). Two separate spots have been observed under UV light for methanol extract of *Macrotyloma uniflorum*. The separated spots on TLC plates indicate presence of different compounds which were further analyzed by GC-MS.

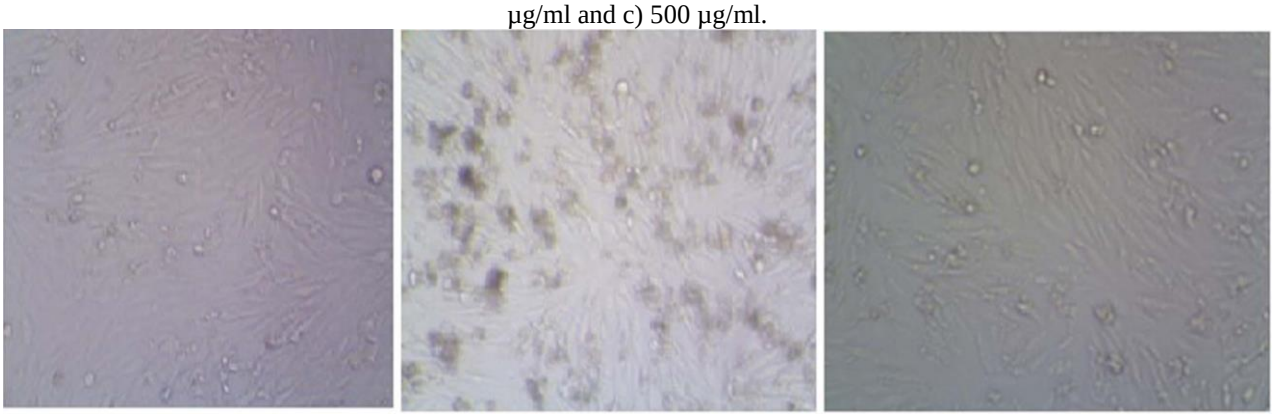
GC-MS

In order to determine the compounds, present in the extract of *Macrotyloma uniflorum*, GC-MS analysis was done. This analysis revealed that both methanol and ethanol extract of *Macrotyloma uniflorum* contain different compounds. Some of them are known for their biological



(A) (B) (C)

Figure 3: anticancer activity of Macrotyloma uniflorum methanol extract, a) cell viability in control sample, b) 250 µg/ml and c) 500 µg/ml.



(A) (B) (C)

Figure 4: anticancer activity of Macrotyloma uniflorum ethanol extract, a) cell viability in control sample, b) 250 µg/ml and c) 500 µg/ml.

activity and a few other compounds remain unexplored. The GC-MS chromatogram of methanol and ethanol extract is presented in fig 2. From GC-MS analysis, it has been seen that Propanedioic acid is present in methanol extract of *Macrotyloma uniflorum*, which is reported to possess antioxidant activity[23] antimicrobial, and antiseptic activity[24]. However, N-(3-methylaminopropyl)-N-methylformamide activity is not known. Ethanol extract of *Macrotyloma uniflorum* contains Hydroxyurea which is reported to have anticancer effect. Propanamide is amide form of propanoic acid, known to possess antioxidant activity and Amino-1-propanol activity is unknown yet. Activities of these compounds are believed to be responsible for the bioactivity of *Macrotyloma uniflorum* extracts.

Antimicrobial Test

Antimicrobial activity of *Macrotyloma uniflorum* was checked against nine clinical pathogens. Antimicrobial activity was determined by measuring zone of inhibition formed after incubation period. The methanol extract showed characteristic zone of inhibition against five pathogens including *Pseudomonas aeruginosa*, *Serretia* sp., *Salmonella* sp. and *Klebsiella* sp. and *Escherichia coli* among nine test pathogens. While, ethanol extract has only shown activity against *Klebsiella* sp and *Proteus* sp, however zone of inhibition was very pronounced (table 2, table 3). Highest zone of inhibition was observed at 100mg/ml concentration against *Pseudomonas aeruginosa*. Methanol extract showed antimicrobial activity against both Gram positive and Gram negative organism

Antioxidant Test

The antioxidant activity of *Macrotyloma uniflorum* extracts was evaluated by DPPH radical scavenging assay which was originally described by Blois[18]. DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a synthetic free radical with deep violet colour when is in form of solution it has a λ_{max} at 517nm. *Macrotyloma uniflorum*, methanol extract showed better antioxidant activity than ethanol extarct (table 4). The reducing power of methanol extract indicates presence of some compounds in *Macrotyloma uniflorum* extracts which can donate electron and react with free radicals to convert them into more stable products and to terminate radical chain reactions. Increased absorbance of reaction mixture indicates increased reducing power of the extract[25].

Anticancer study

Anticancer study of the *Macrotyloma uniflorum* was done against human osteosarcoma cell line (MG 63). Anticancer activities of methanol and ethanol extract were checked. The activity of *Macrotyloma uniflorum* methanol and ethanol was shown in fig 3 and fig 4 respectively. In methanol extract cell viability is decreased with increased concentration of extract, which indicates the moderate activity of the extract. At 200 µg/ml concentration methanol and ethanol extract has shown 82% and 74% cell

Table 4: Antioxidant activity of Macrotyloma uniflorum methanol and ethanol extract.

Sample	OD Sample At 517nm	Percentage Of Inhibition
Standard	0.823	
Methanol extract	0.456	44%
Ethanol extract	0.312	62%

viability[26]. The data indicates the presence of anticancer activity of both methanol extract of *Macrotyloma uniflorum*.

CONCLUSION

Based on thepresent work, this study shows that the methanol and ethanol extract of *Macrotyloma uniflorum* exhibited significant antimicrobial, antioxidant and anticancer activity. It can be used as alternative crop in traditional diet as a beneficial source of food with very high nutritional value and support the concept of functional foods. These insights may also aid in the use of this legumes as ingredients in composite legume flours and functional food product development for dietary management of diabetes and related hypertension.

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