https://doi.org/10.30799/jnpr.063.18040203 J. Nat. Prod. Resour. - Volume 4 Issue 2 (2018) 185–187

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ISSN: 2455-0299

Phytochemical Studies on *Theriophonum minutum* Extracts and Evaluation of Its Anticancer Activity

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ARTICLE DETAILS

Article history:
Received 12 June 2018
Accepted 26 June 2018
Available online 07 July 2018

Keywords:
Theriophonum minutum
Allium cepa Assay
Yeast Proliferation Model
SRB Assay

ABSTRACT

The present investigation deal to phytochemical studies of *Theriophonum minutum* (TM) extracts and evaluation of its anticancer potential. The dried plant of TM was successively extracted with petroleum ether, ethyl acetate, ethanol, hydro-alcoholic and water. Various extracts of TM reveal the presence of sterols, flavonoids, alkaloids, glycoside, etc. TM extracts elicited significant *in vitro* antimitotic and antiproliferative assay *by Allium cepa* root inhibition and yeast proliferation model respectively. Ethyl acetate TM extract (200 mg/mL) exhibited interruption in cell proliferation of yeast as observed by marked reduction in number of dividing cells and inhibition of cell viability compared to control. Ethanolic extract of TM (100 mg/mL) has demonstrated significant inhibitory root growth in *Allium cepa* model. Therefore it was further evaluated by sulforhodamine B (SRB) assay in cell culture of human prostate (PC3), colon cancer (Colo-205) cell lines. Despite of significant antimitotic and antiproliferative activity, ethanolic TM extract exhibited low cytotoxicity in SRB assay.

1. Introduction

Ethanopharmacological properties of the herbs have been used as primary source of medicines for early drug discovery. According to WHO, 80% of the people still rely on plant based traditional medicines for primary health care [1]. The knowledge associated with traditional medicines has promoted further investigations for medicinal plants as potential medicines that has led to the isolation of many natural products that have become well known pharmaceuticals [2].

The National Cancer Institute (NCI) has several ongoing collaborative programs which screen plant derived extracts, fractions and isolated constituents for the possibility of new drugs. There are many phytochemicals which are still under clinical trial for cancer such as calanolide A and B (costatolide), conocurovone, perillyl alcohol and flavopiridol, 9-aminocamptothecine [3]. Seven plant derived anticancer drugs have received FDA approval for commercial production includes taxol, paclitaxel, vinblastine, vincristine, topotecan, irinotecan and teniposide [4].

The genus Theriophonum (Araceae), represented by seasonally dormant tuberous perennials is endemic to India and Sri Lanka [5]. Literature review illustrates that various herbs from the family Araceae exhibited anticancer property like *Colocasia esculenta* (Linn.), *Acorus calamus* [6] resulted in moderate anticancer activities against MCF-7 and HT-29 cell lines [7]. *Theriophonum minutum* is a wild edible plant show naturally variability and contains relatively higher nutritive values compare to conventional foods resources [8]. Extensive literature survey reveals that *Theriophonum minutum* has not explored in terms of its phytochemical profile as well as its pharmacological activity, although it has been reported for its excellent nutritive values. Hence, the present investigation is to explore medicinal properties of *Theriophonum minutum* to base on its phytochemical profile.

2. Experimental Methods

2.1 Collection and Identification of Plant Material

The plant of *Theriophonum minutum* were collected from Salakasa taluka belongs to Deori sub division of Gondia district, Maharashtra, India. The herbarium specimen was authenticated at Department of Botany, RTM Nagpur University and it was noted as Specimen Voucher no.10100. The dried plant were pulverized into course powder. About 500 g of powered crude drug defatted with petroleum ether and successively extracted with ethyl acetate, ethanol, hydro-alcoholic and water.

2.2 Phytochemical Screening

Preliminary phytochemical screening of the *Theriophonum minutum* extracts were carried out to identify the presence of various secondary metabolites [9].

2.3 Thin Layer Chromatography (TLC)

Theriophonum minutum extracts were subjected to thin layer chromatography to determine number and nature of phytoconstituents in each extract [10].

2.4 Antiproliferative Activity

2.4.1 Yeast Proliferation Model [11]

Potatoes dextrose broth 2.5 g was dissolved in 100 mL distilled water. It was sterilized by autoclaving at 121 °C, 15 lbs pressure for 20 min. Inoculum of yeast was done on flask containing 100 mL sterilized broth. It was incubated at 37 °C for 24 h. This is referred as seeded broth. Further test inoculum was prepared by mixing 1 mL of seeded broth with 10 mL sterilized distilled water (25.4×10^4 cells/mL). A solution of *Theriophonum minutum* extracts of concentration 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL were prepared. To determine cell viability count, solution containing 2.5 mL of sterilized potato dextrose broth and 0.5 mL of yeast inoculum were added in six separate test tubes. The first tube was kept as control without extract. In the second, third and fourth tubes, 1 mL of extract at concentration of 5, 10, 15 and 20 mg/mL were added while in fifth and sixth tube standard anticancer drug methotrexate (50 µg/mL) was added. All tubes were incubated for 24 h at 37 °C. The cell viability count was done using methylene blue differential staining. In the above suspension, 0.1% methylene blue dye was added in all tubes and was observed under low power microscope. Viable cells remain unstained while dead cells were stained blue in color.

The number of viable cells was counted in 16 chambers of hemocytometer and the average number of cells was calculated. The cells per mL and percentage of cell viability and percent inhibition of cell viability were calculated by following formulae,

Viable cells/mL = average no. of viable cell in one square x dilution factor x 10^4

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https://doi.org/10.30799/jnpr.063.18040203

Cite this Article as: Ashwini Khubalkar, Amol Warokar, Radheshyam Lohiya, Milind Umekar, Phytochemical studies on *Theriophonum minutum* extracts and evaluation of its anticancer activity, J. Nat. Prod. Resour. 4(2) (2018) 185-187.

$$\% \text{ Cell viability } = \frac{\text{Total viable cells}}{\text{Total number of cells}} X\ 100$$

$$\% \text{ Inhibition of Cell viability} = \frac{\text{Cell viability control} - \text{Cell viability treated}}{\text{Cell viability of Control}} X\ 100$$

2.4.2 Antimitotic Activity [12]

Onion bulbs (50±10 g) were procured from local market and grown in the dark over 50 mL tap water at ambient temperature until the roots have grown to approximately 2-3 cm length. The base of each of the bulbs was immersed on the 50 mL extract of *Theriophonum minutum* of concentration 100 mg/mL. Root length (newly appearing roots not included) and root number at 0, 48 and 72 h for each concentration of extract and control were measured. The percentage root growth inhibition after treating with test extract at 48 and 72 h were determined. Methotrexate (standard) as well as extract of roots was used at 10 mg/mL concentration. The extract of *Theriophonum minutum* produced dose and time dependent growth inhibition. Incubation of bulbs in different concentrations of extract and standard produced a growth retarding effect that was associated with a decrease in the root number and shrinkage of existing roots. Onion bulbs showing the effect of different extract of roots of *Theriophonum minutum* on root length following 72 h of incubation.

2.4.3 Anticancer Activity

Anticancer activity was evaluated by sulforhodamine B (SRB) assay in cell culture of human prostate (PC3) and colon cancer (Colo-205) cell lines. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mm L-glutamine. Cells were inoculated into 96 well microtiter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5 % CO_2, 95% air and 100% relative humidity for 24 h prior to addition of *Theriophonum minutum* extracts. Test extracts were initially solubilized in dimethyl sulphoxide at 100 mg/mL. The dilution were made to obtain concentration of 1 mg/mL using water and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate (1 mg/mL) was thawed and diluted to 100, 200, 400 and 800 µg/mL with complete medium. Aliquots of 10 µL of these different *Theriophonum minutum* extracts dilutions were added to the appropriate microtiter wells already containing 90 µL of medium, resulting in the required final drug concentrations i.e.10, 20, 40 and 80 µg/mL.

After addition of test extract, plates were incubated at standard conditions for 48 h and assay was terminated by the addition of cold trichloroacetic acid (TCA). Cells were fixed *in situ* by the gentle addition of 50 µL of cold 30% (w/v) TCA and incubated for 60 minutes at 4 °C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µL) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by rinsing with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mm trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells x 100. Using the six absorbance measurements [time zero (Tz), control growth (C), and test extract growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as: [Ti/C] x 100%.

3. Results and Discussion

3.1 Phytochemical Screening

Theriophonum minutum revealed the presence of flavonoids, sterols, glycoside, alkaloids, terpenoids, fats and oil, gum and mucilage and phenolic compounds. The results of phytochemical studies of various extracts of *Theriophonum minutum* are shown in Table 1.

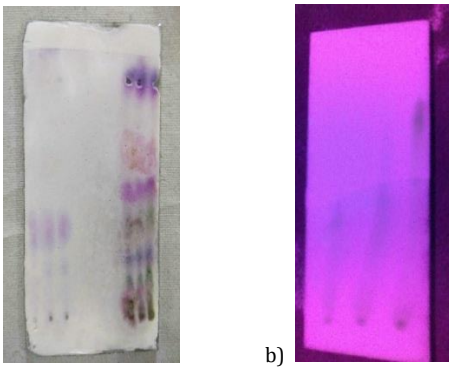
3.2 Thin Layer Chromatography (TLC)

Theriophonum minutum plant extracts were evaluated to thin layer chromatography to determine number of phytoconstituents and nature of components in each extracts. TLC ethyl acetate plant extract *Theriophonum* minutum (EATM) was optimized in mobile phase toluene: chloroform: formic acid (5:4.5:0.5) where vanillin-H_2SO_4 was used as spraying reagent (Fig. 1). TLC ethanol extract *Theriophonum minutum* (ETTM) was optimized in mobile phase butanol: acetic acid: water (4:2:2) where H_2SO_4 was used as spraying reagent (Fig. 1) subsequently TLC was observed under fluorescent background.

Table 1 Preliminary phytochemical screening of *Theriophonum minutum* extracts

Plant Constituents	Test/Reagent	Extract			
		PE	EA	ET	HA
Sterols	Salkowaski	+	+	+	+
	Libermann-Burchard	+	+	+	+
Alkaloids	Dragendorff's	–	–	+	+
	Hager's	–	–	+	+
	Mayer's	–	–	+	+
	Wagner's	–	–	+	+
Saponins	Foam	–	–	–	+
Glycoside	Keller-killiani	+	+	+	+
	Legal test	+	+	+	+
	Bortrager's	+	+	+	+
	Coumarin glycoside	–	–	–	+
Phenols & Tannins	Ferric Chloride	–	–	+	+
	Lead acetate	–	–	+	+
	Pot. Dichromate	–	–	+	+
Flavonoids	Shinoda	–	+	+	+
Carbohydrates	Molisch	–	–	+	–
	Fehlings	–	–	+	–
	Barfoed's	–	–	+	–
Terpenoids	1% $Cuso_4$	–	+	+	+
	Millon's	–	–	–	–
Protein	Biuret	–	–	+	+
	Xanthoproteic	–	–	+	+
fats or oil	Fixed oil	+	+	+	+
Gum and Mucilage	Absolute alcohol	+	+	+	+

(+) = Present, (–) = Absent



a) b)

Fig. 1 TLC of a) ethyl acetate and b) ethanol plant extract of *Theriophonum minutum*

Table 2 Percentage inhibition of cell viability by *Theriophonum minutum* on yeast model

S. No.	Sample mg/mL	Total no. of viable cells	Total no. of cells	% cell viability	% inhibition of cell viability
Control	-	482	529	91	-
Standard	50 µg/mL	82	97	84.53	82.98
Ethyl acetate	5	207	517	40.03	57.05
	10	189	450	42	60.78
	15	98	425	23.05	79.66
	20	84	432	19.44	82.57
Ethanol	5	188	435	43.21	60.99
	10	144	427	33.72	70.12
	15	109	412	26.45	77.38
	20	98	402	24.37	79.66
Hydro-alcoholic	5	243	438	55.47	49.58
	10	148	441	33.56	69.29
	15	121	439	27.56	74.89
	20	99	438	22.60	79.46

3.3 Yeast Proliferation Model

The antiproliferative potential of *Theriophonum minutum* plant extract by using *Saccharomyces cerevisiae* (yeast) model showed that test extracts were inhibitor of cell growth and the inhibition in proliferation of yeast were dose dependent with increasing concentration of the extract. The inhibition of viable cells were superior in ethyl acetate extract 82.57% at 20 mg/mL of *Theriophonum minutum* which was near to the standard anticancer drug methotrexate (82.98%) at 50 µg/mL. Percentage

https://doi.org/10.30799/jnpr.063.18040203

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inhibition of cell viability on yeast by various extracts of *Theriophonum minutum* have shown in Table 2.

3.4 Mitotic Cell Division in Allium cepa Assay

Effect of test extract of *Theriophonum minutum* plant and standard methotrexate was studied on root growth *Allium cepa* root. *Allium cepa* root meristematic cells as indicated by inhibition of root growth (reduced root length) and decreased mitotic index after 72 h of treatment. The maximum inhibition of root growth was observed at 100 mg/mL affecting the rootlet morphology (shrinking of the rootlets and dark brown color observed in ethanol extract). Ethanol extracts of *Theriophonum minutum* plant (ETTM) at 100 mg/mL exhibited inhibitory significant influence on root growth compared to control group. The inhibitory effect of various extracts of *Theriophonum minutum* plant are shown in Table 3. Ethanol extracts of *Theriophonum minutum* plant has shown inhibitory effect on growth of roots of *Allium cepa* (Fig. 3).

Table 3 Effect of *Theriophonum minutum* on *Allium cepa* root growth

S. No.	Groups	Concentration mg/mL	Root length (cm)		
			0 h	48 h	72 h
1	Control (Tap water)	-	1.1	2.3	2.1
2	Ethyl acetate	100	1.2	3.3	3.5
3	Ethanol	100	1.1	3.7	3.2
4	Hydro-alcoholic	100	1.3	3.9	3.6
5	Standard (Methotrexate)	50 µg/mL	1.4	3.5	3.3

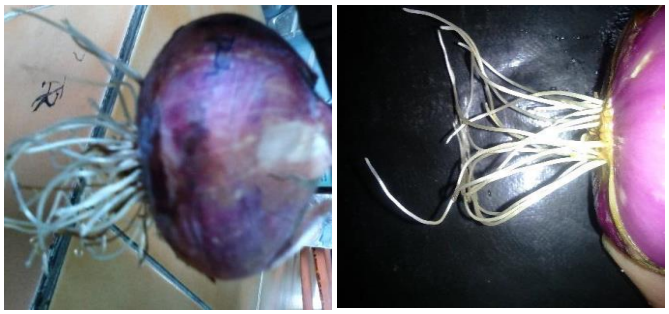

Fig. 3 (A) Non treated (B) Onium root treated by onium root ETTM (100 mg/mL) after 72 h

3.5 Anticancer Activity

It was evaluated by sulforhodamine B (SRB) assay in cell culture of human prostate (PC3) and colon cancer (Colo-205) cell lines. The anticancer potential exhibited by various extracts of *Theriophonum minutum* plant are shown in Tables 4 and 5.

Despite of promising antiproliferative activity, *Theriophonum minutum* plant extracts elicit lower anticancer activity. Inadequate amount of phytoconstituents in the crude extract might be the reason for lower anticancer effect. Therefore bioactivity guided fractionation, isolation of specific phytoconstituents and its subsequent screening may be futuristic approach of present investigation.

Table 4 Anticancer activity responds to human prostate (PC-3) cell line

	Drug Concentration (µg/mL)														
	Experiment 1				Experiment 2				Experiment 3				Calculated from graph		
PC-3	10	20	40	80	10	20	40	80	10	20	40	80	LC50	TGI	GI50*
EA-1	97.5	102.4	99.7	91.5	94.7	106.0	94.0	87.2	99.8	104.4	111.2	72.5	NE	NE	NE
ET-1	92.5	102.5	98.8	105.1	97.7	101.7	102.3	100.8	95.1	95.6	103.7	87.6	NE	NE	NE
HA1	104.0	105.0	103.8	107.2	99.5	110.0	104.3	105.7	88.9	101.4	103.9	93.1	NE	NE	NE
ADR	-44.9	-52.4	-58.1	-49.1	-43.9	-49.5	-58.7	-46.0	-43.3	-52.5	-52.6	-50.0	<10	<10	<10

Table 5 Anticancer activity responds to human colon cancer (Colo-205) cell line

	Drug Concentration (µg/mL)														
	Experiment 1				Experiment 2				Experiment 3				Calculated from graph		
Colo-205	10	20	40	80	10	20	40	80	10	20	40	80	LC-50	TGI	GI50*
EA-1	66.9	59.4	70.9	66.9	77.0	78.3	78.7	80.6	81.9	81.6	83.4	86.8	NE	NE	NE
ET-1	63.0	69.2	68.5	69.1	76.9	76.0	78.4	82.2	80.8	82.5	82.0	87.9	NE	NE	NE
HA-1	62.5	69.3	71.4	73.1	77.0	79.2	84.1	85.9	78.1	79.1	82.7	88.8	NE	NE	NE
ADR	2.1	-2.0	-19.8	-6.9	1.6	4.7	-12.1	4.3	7.1	0.9	-16.8	-2.3	NE	<10	<10

4. Conclusion

Despite of significant antimitotic and antiproliferative activity, ethanolic *Theriophonum minutum* extract exhibited low cytotoxicity in SRB assay. The phytoconstituents need to be isolated from the extracts and further screen for anticancer activity.

Acknowledgement

The authors are thankfull to Tata Memorial Cancer Hospital, Mumbai, Maharashtra, India, for screening the test extracts for SRB assay.

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https://doi.org/10.30799/jnpr.063.18040203

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