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TO EVALUATE ANTIMITOTIC AND ANTIPROLIFERATIVE ACTIVITY OF EXTRACTS OF *C. TERNATEA*

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ABSTRACT

The works aims to evaluate antimitotic and antiproliferative activity of extracts of *C. Ternatea*. The work exploits the antioxidant potential using DPPH radical Scavenging method and nitric oxide radical scavenging method. The antimitotic activity was screened using *Allium cepa* root meristamatic cells which have been used extensively in screening of drug with antimitotic activity. The anti-proliferation activity was evaluated using Yeast as a model system.Percentage of Mitotic index for the test extracts ACCT and MECT was found to be 35.84 % and 46.87 % respectively. ACCT was found to be most potent than other extracts and also appeared significantly better than that of standard, Methotrexate with 43.05 %. The anti-proliferative activity of the selected extracts was observed in decreasing order of ACCT > MECT. ACCT appeared most potent with 49.60 % and MECT with 30.56 % inhibition respectively at 100 mg/ml. The promising antimitotic and anti-proliferative activity of ACCT can be directly correlated with the presence of higher amount of total flavonoids and polyphenols. It is evident from these studies that successive extraction of these plants lead to segregation of biomolecules and degree of polymerization plays an important role in determining the pharmacological potential of extracts and isolated molecules.

KEYWORDS: *C. ternatea*, flavonoids, DPPH and nitric oxide.

INTRODUCTION

Antioxidants are found in a wide variety fruits and vegetables, plant extracts, beverages, herbs and spices and semisynthetics. They have been found to inhibit various types of cancers. One of the most important contributions to cancer is considered to be oxidative

damage to DNA. If a cell containing damaged DNA divides before the DNA can be repaired, the result is likely to be a permanent genetic alteration of the steps in carcinogenesis. Body cells that divide rapidly are more susceptible to carcinogenesis because there is less opportunity for DNA repair before cell division. There is compelling evidence that antioxidants and antiinflammatory compounds (including anti-iron and anti-copper compounds) could be used to modify the redox environment of cancer cells and thus their behavior.[1]

Flavonoids are the water-soluble pigments in vegetables, fruits, grains, flowers, leaves and bark. These pigments can scavenge superoxide, hydroxy and proxyradicals, breaking lipid peroxide chain reactions. They have also been shown to protect cells from X-ray damage, to block progression of cell cycle, to inhibit mutations, to block prostaglandin synthesis, and to prevent multistage carcinogenesis in experimental animals [Abdulla, 2000].[2] Based on these results, flavonoids may emerge as promising anticancer agents (Kanadaswami et al. 2005).[3] Experimental animal studies indicate that certain dietary flavonoids possess antitumor activity. The hydroxylations pattern of the B ring of the flavones and flavonols, such as luteolin and quercetin, seems to critically influence their activities, especially the inhibition of protein kinase activity and antiproliferation (Abu-Dahab and Afifi, 2007).[4] Although the mechanism by which flavonoids exert their actions is not totally clear, the ability to modulate protein phosphorylation is considered an essential feature (Chen et al., 2004; Korkina and Afanas'ev, 1997).[5,6]

MATERIAL AND METHODS

Procurement and Authentication

The leaves of *Clitoria ternatea* Linn were collected locally from Nagpur. The plants were authenticated by botanist, Dr. Alka Chaturvedi, Department of Botany, R. T. M. Nagpur University Campus, Nagpur.

Extraction

The air-dried parts of the selected plants were pulverized into a coarse powder. The powdered crude material (1 kg) was defatted with petroleum ether (60-80°C) in Soxhlet apparatus. The defatted crude material was then extracted successively with chloroform, ethyl acetate, acetone and methanol using Soxhlet apparatus and finally macerated with hydroalcoholic solvent. All the above extracts were concentrated in a rotary vacuum evaporator (Sonar,

India) to yield a dark brown syrupy mass. The quantity of extract (practical yield) so obtained, from leaves of *Clitoria ternatea* is tabulated in Table 1.

Table 1: Amount of extracts obtained from leaves of *Clitoria ternatea*

Plant	Type of Extract	Appearance/ State	Yield (% w/w)
Leaves of *Clitoria ternatea*	Petroleum Ether	Dark Brown/ Semisolid	1.9
	Chloroform	Dark Brown-black/ Semisolid	2.2
	Ethyl acetate	Dark Brown-black/ Semisolid	4.5
	Acetone	Dark Brown-black/ Semisolid	5.5
	Methanolic	Dark Brown-black/ Semisolid	15.6
	Hydroalcoholic	Dark Brown-black/ Semisolid	16.1

Preliminary phytochemical screening[9]

All the Extracts were subjected to Phytochemical screening for observation of active constituents present in extracts as shown in Table 2.

Extracts of *Clitoria ternatea* were represented as, PECT: Petroleum ether ex tract; CHCT: Chloroform extract; EACT: Ethyl acetate extract; ACCT: Acetone extract; MECT: Methanol extract and HACT: Hydroalcoholic extract

Table 2: Preliminary phytochemical screening of extracts of *Clitoria ternatea* leaf

Chemical tests	***Plant parts used***					
	Clitoria ternatea leaf extracts					
	Name of the extracts					
	PECT	**CHCT**	**EACT**	**ACCT**	**MECT**	**HACT**
Proteins & Amino acid	-	-	-	+	+	+
Carbohydrate	-	-	-	-	+	+
Sterol	+	+	-	-	-	-
Terpenoids	+	+	-	-	-	-
Triterpenoids	-	-	-	-	+	-
Saponin	-	-	-	-	-	-
Flavonoid	-	-	+	+	+	+
Alkaloid	-	-	-	-	+	+
Tannin	-	-	-	+	+	+

+: Present and -: Absent.

Determination of physicochemical parameters

The crude drugs were grinded in a mixer grinder and were further used for evaluation of various physicochemical parameters such as different ash values such as total ash, acid insoluble ash and water soluble ash values were determined by incinerating the plant material at a temperature between 500-600˚C until it is white, indicating the absence of carbon.

Extractive values in different solvents of the crude drugs were evaluated by soaking it in respective solvents for about 18 h. Physicochemical parameter like water soluble extractive value, alcohol soluble extractive value, total ash value and acid insoluble ash of crude material were determined shown in Table 3.

Table 3: Ash and Extractive value of leaves of *Clitoria ternatea*

Sr. No.	Parameters		leaves of *Clitoria ternatea*
1.	**Ash value (% w/w)**	Total ash	8.95
		Acid insoluble ash	0.62
2.	**Extractive value (% w/w)**	Ethanol soluble	7.22 %

Quantitative estimations

The total phenolic (TP) content (mg/g) in extract and its fractions were determined from regression equation of calibration curve of gallic acid (y = 0.005x + 0.075, r^2= 0.998) and expressed as GAE of extract (Table 9). While, Flavonoid content (TFA) was determined from linear regression equation of Rutin (y= 0.018x - 0.001, r^2 = 0.990). The flavanone content (TFO) determined by 2, 4-dinitrophenylhydrazine was calculated from linear regression equation of Naringin (y= 0.006x + 0.020, r^2 = 0.995). The flavonoid and flavanone content represented only 12.91% (w/w) and 2.87 % (w/w) of the TP in HACT extract, respectively as shown in Table 4.

Table 4: Total Polyphenol, flavonoid, flavanone, total flavonoid content and degree of polymerization of extracts of leaves of *Clitorea ternatea*

Sr. No.	Test Sample	Total Polyphenol Content (TP) (GAE mg/g of extract)	Flavonoid Content (TFA) (RE mg/g of extract)	Flavanone Content (TFO) (NE mg/g of extract)	Total Flavonoid Content (TF)#	Degree of polymerization
1.	EACT	8.4487 ± 0.105*	1.996 ± 0.028**	0.666 ± 0.18*	2.662	4.2322
2.	ACCT	35.159 ± 0.093**	4.582 ± 0.287*	1.238 ± 0.271*	5.821	7.6732
3.	MECT	41.381 ± 0.504	4.121 ± 0.103*	1.313 ± 0.014**	5.434	10.0405
4.	HACT	22.506 ± 0.676	4.632 ± 0.17*	0.856 ± 0.079**	5.494	4.8510

Results are mean ± SD of three replicates: GAE, RE and NE: gallic acid, Rutin and Naringin equivalents, respectively. The estimation of the degree of polymerization is calculated by the ratio between TP and TFA; # Total flavonoid content is determined by adding flavonoid content with flavanone content; *Represents statistical significance ($p < 0.05$). **Represents Statistical significance($p<0.001$)

HPTLC Study

The identification of major groups was carried out by TLC and HPTLC as shown in Table 5.

Table 5 -Mobile phase for HPTLC studies of extracts of *Clitoria ternatea*

Sr. no.	Test extract	Solvent system	Number of Spots
01	PECT	Toluene: Chloroform(7:3)	05
02	CHCT	Toluene: Methanol(8.5:1.5)	06
03	EACT	Ethyl Acetate : Methanol(9:1)	06
04	ACCT	Ethyl Acetate : Methanol(7:3)	07
05	MECT	Ethyl Acetate: Methanol: Triethylamine (7:2.5:0.5)	07
06	HACT	Ethyl Acetate: Methanol: Glacial acetic acid (6.5:2.7:0.8)	09

Antioxidant activity

Antioxidant activity of all the selected extract has been determined by DPPH assay and Nitric Oxide Radical Inhibition Activity. The most significant antioxidant activity was exhibited by the MECT extract. The highest NO radical scavenging activity was observed in ACCT extract, as shown in Table 6.

Table 6: Antioxidant activities of extracts of aerial parts of *Clitoria ternatea*

Test Samples	IC_{50} value of DPPH assay (as µg/ml)	IC_{50} value of NO assay (as µg/ml)
Ascorbic acid	2.2913	8.1445
Gallic Acid	1.40	5.9654
Rutin	2.1120	19.6658
EACT	41.53142	525.9544
ACCT	19.01588	529.1405
MECT	13.80568	777.9656
HACT	47.054429	793.0282

Antimitotic Activity[10]

The antimitotic activity was screened using *Allium cepa* root meristamatic cells which have been used extensively in screening of drug with antimitotic activity as shown in Table 7.

Table 7: *Allium Cepa,* Comparison between Mitotic Index with Water, Methotrexate, ACCT and MECT extracts

Sample	Total no. of cells	No. of cells in mitosis	Mitotic index (%)
Water	96	64	66.66
Methotrexate	72	31	43.05
ACCT	53	19	35.84
MECT	32	15	46.87

Anti-proliferative Activity[11]

Anti-proliferative study was carried out by using yeast as a model system.

Anti-proliferation study: Effect of different concentration of **ACCT** extract on yeast cell growth as shown in Table 8 and Table 8.

Table 8: Concentration and Number of Viable Cells

Extract	Concentration in (mg/ml) and number of viable cells per ml				
	20	40	60	80	100
ACCT extract	221000	203000	189000	155000	127000
MECT extract	235000	228000	212000	198000	175000

$$\text{Percentage inhibition of growth} = \frac{\text{Viable cells in control - Viable cells in test}}{\text{Control}} \times 100$$

Table 9: Percentage Growth Inhibition of Yeast Cells

Extract	Concentration in (mg/ml) and % Growth inhibition of viable cells					IC_{50} (mg/ml)
	20	40	60	80	100	
ACCT	12.30	19.44	25.00	38.49	49.60	100.80
MECT	6.75	9.52	15.87	21.43	30.56	163.64

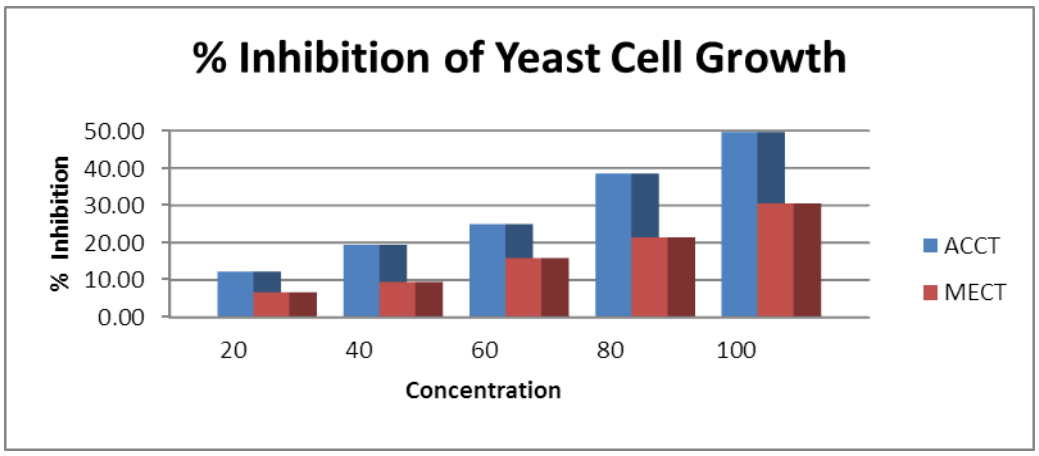

Figure 1: Percentage Inhibition of Yeast Cell Growth

RESULTS AND DISCUSSION

The present work deals with the study of leaves of *Clitoria ternatea* for the macro and microscopic characterization and determination of its physicochemical parameters, the qualitative and quantitative analysis of the secondary metabolites and their HPTLC fingerprinting.

Physicochemical studies were carried out, as per the Indian and Ayurvedic Pharmacopoeia of India such as total ash, acid insoluble ash value, water soluble and ethanol soluble extractive values. Ash values are important quantitative standards, which showed the presence of inorganic salts either naturally occurring or adhering to it, or deliberately added to it as a form of adulteration. While, the extractive values are indicative of approximate measures of their chemical constituents, these values are used to determine the quality of drugs since long times. Drugs are identified as of substandard quality due to either faulty collection or incorrect storage.

All the above extracts were concentrated in a rotary vacuum evaporator to yield a dark brown syrupy mass. Extracts of *C. ternatea* were represented as PECT: Petroleum ether extract; CHCT: Chloroform extract ; EACT: Ethyl acetate extract ; ACCT: Acetone extract; MECT: Methanol extract and HACT: Hydroalcoholic extract.

Preliminary Phytochemical screening of the *C. ternatea* extracts were carried out for the presence of various important secondary metabolites (Harbone, 1976; Stahl, 1969; Wagner et al., 1984**).** Amongst the *C. ternatea* extracts, PECT and CHCT extracts showed the presence of sterol and terpenoids, EACT showed presence of flavonoid, ACCT showed presence of proteins and amino acids, flavonoid and tannins, MECT showed presence of proteins, amino acids, carbohydrate, triterpenoids, flavonoid and tannins. HACT showed presence of proteins, amino acids, carbohydrate, flavonoid, alkaloid and tannins.

The observation from the quantitative estimation of secondary metabolites such as the total phenolic (TP) content (mg/g) was determined by Folin Ciocalteu method, Flavonoid content (TFA) was determined by the aluminium chloride method (Jin-Yuarn and Ching-Yin, 2007; Stanojević et al., 2009) and the modified 2, 4-dinitrophenylhydrazine (DNPH) method was used for determination of flavanones (TFO) (Nagy and Grancai, 1996). The contents determined by these two methods were added up to obtain the total flavonoid content (Chang et al., 2002). The results of this study suggested that total phenolic (TP) content (mg/g) in

extracts were determined and expressed as Gallic acid equivalent (GAE/g) of the extract. While, Flavonoid content (TFA) was determined from linear regression equation of rutin and expressed as Rutin equivalent (RE/g) of the extract. The flavanone content (TFO) was determined by 2, 4-dinitrophenylhydrazine method and expressed as Naringin equivalent (NE/g) of the extract. The flavonoid and flavanone content represented only 12.91% (w/w) and 2.87 % (w/w) of the TP in HACT extract respectively and similar pattern was observed in all its fractions, suggesting that the extracts are very complex, and contain many other polyphenols such as flavanones, isoflavones, phenolic acids and tannins, and the degree of polymerization of the polyphenols present in the samples is high. Degree of polymerization was estimated by the ratio between the TP and TFA contents (Souza et al., 2008). Amongst the extracts of C. *ternatea*, highest total polyphenol content and flavonoid content was observed in MECT and ACCT extract respectively. However, highest flavanone content was observed in MECT extract. Results obtained from experiment revealed that, the extracts have shown polyphenolic content in decreasing order of MECT > ACCT > HACT > EACT and flavonoid content in the decreasing order of HACT > ACCT > MECT > EACT respectively. On the other hand, degree of polymerization was observed in the decreasing order of MECT > ACCT > HACT > EACT extract respectively. All the extracts were subjected to chromatographic (HPTLC) profiling to estimate the number of phytoconstituents. Numbers of solvents systems were developed and tried for all the extracts. However, the solvent system which gave best resolution was considered valid and useful. The HPTLC fingerprints of all the extracts of both the plants under study may therefore be able to work as guidelines for standardisation of these plants in future.

Antioxidant study of all the plant extracts were determined by two different methods, since the results of the determination of the antioxidant capacity of an extract depends greatly on the methodology used, specifically, the oxidant and the oxidizable substrate used in the measurement. Therefore, it is important to compare different analytical methods varying in their oxidation initiators and targets in order to understand the biological activity of an antioxidant (Cao and Prior, 1998). The antioxidant activity of the plant extracts was assessed based on radical scavenging effect of the stable DPPH free radical (Sarikurkcu et al., 2008; Scherer and Godoy, 2009; Sharma and Bhat, 2009) and by Nitric oxide assay wherein, nitric oxide was generated from sodium nitroprusside and measured by the Griess-llosvog Reaction (Garrat, 1964).

Results from the experiment using DPPH method revealed that, amongst the extracts of *C. ternatea,* the MECT extract which is high in flavonoid and polyphenol content has shown lowest IC_{50} value followed by the flavonoid rich ACCT extract. Results clearly indicated that alongwith flavonoid and polyphenols other phytoconstituents such as triterpenoids present in MECT extract may remain responsible for its antioxidant activity. The DPPH radical scavenging activity was observed in decreasing order of MECT > ACCT> EACT> HACT. The potent antioxidant potential of ACCT extract was due to the presence of higher amount of total flavonoid and polyphenols compared to other extract of *C. ternatea*. On the other hand, higher antioxidant potential of EACT extract than the HACT extract, which contains higher amount of polyphenols and flavonoid, may be because of its steroidal content and other secondary metabolites present in it. However, by NO assay, the highest NO radical scavenging activity was observed in ACCT extract. The most potent antioxidant potential of ACCT extract may be attributed to the presence of higher amount of total flavonoid and polyphenols compared to other extracts. The NO radical scavenging activity was observed in decreasing order of ACCT > EACT > MECT > HACT. Thus, it is evident that successive extraction has resulted in segregation of compounds with high antioxidant potential.

Results from the antioxidant activity experiment revealed that, antioxidant activity of different extracts and the quantitative evaluation for the targeted bioactive constituents such as polyphenols, optimised the best two extracts from this drug i.e. MECT and ACCT extract of *Clitoria ternatea* were selected further studies on its antimitotic and antiproliferative potential.

The pharmacological screening of the selected extracts i.e ACCT and MECT of *C. ternatea* was carried out to evaluate its anti-mitotic and anti-proliferative activity. The antimitotic activity was screened using *Allium cepa* root meristimatic cells, which have been used extensively in screening of drug with antimitotic activity (Pardesi et al., 2008). It has been observed that as compared to control, all the extracts showed antimitotic activity. The extract ACCT was found to be most potent than other extracts. % Mitotic index for the test extracts was found to be in the decreasing order of ACCT > MECT. ACCT was found to be most potent than other extracts and also appeared significantly better than that of the standard, Methotrexate.

The anti-proliferation activity was evaluated using Yeast as a model system. The target identification in yeast has proven especially relevant for cancer, which at the simplest level is

a disorder of proliferation control caused by accumulated mutations. The high degree of similarity and function in comparison with human has made Yeast as a model system for anti-proliferative drug discovery **(**Yiannis, 2003). The % inhibition of growth was calculated taking into account the viable cells in control and test extracts. ACCT appeared most potent with 49.60 % inhibition at 100 mg/ml.

Different mechanisms have been linked to flavonoid-mediated cytotoxicity, including proteasome inhibition (D. Chen et al., 2005; A. Kazi et al., 2004), inhibition of fatty acid synthesis (K. Brusselmans et al., 2005), topoisomerase inhibition (A. Constantinou et al., 1995), inhibition of phosphatidyl-inositol 3-kinase (G. Agullo et al., 1997), induction of cell cycle arrest (D.M. Lepley et al., 1996), accumulation of p53 (B. Plaumann et al., 1996) or enhanced expression of c-fos and c-myc (Z.P. Chen et al., 1998). As multiple mechanisms account for flavonoid-induced cytotoxicity, the development of structure–activity relationships to predict the cytotoxic potential of a given compound may facilitate the search for effective candidates for cancer therapy.

Flavonoids are not only being discussed as candidates for cancer therapy but also to play a role in prevention of cancer. There is accumulating evidence that a diet rich in flavonoids is associated with a reduced cancer risk. Many mechanisms of action have been proposed for flavonoid mediated prevention of cancer including estrogenic/anti- estrogenic, antiproliferative effects, antioxidative effects, induction of detoxification enzymes, regulation of the host immune system, changes in cellular signaling and induction of apoptosis (D.F. Birt et al., 2001).[12]

CONCLUSION AND FUTURE SCOPE

The main objective of the present study as already been conversed in the preceding chapters, is to study plants *Clitoria ternatea* for secondary metabolites, antioxidant potential, exploring active extracts followed by evaluation of their antimitotic and antiproliferative potential.The HPTLC fingerprints of all the extracts of both the plants under study may therefore be able to work as guidelines for standardisation of these plants in future. MECT and ACCT extract of *Clitoria ternatea* were selected for further pharmacological studies on the basis of their higher antioxidant potential. The most potent antioxidant potential of these extracts can be attributed to the presence of higher amount of total flavonoid and polyphenols compared to other extracts. It is also evident from these studies that successive extraction of these plants lead to segregation of biomolecules and degree of polymerization plays an important role in

determining the pharmacological potential of extracts and isolated molecules.The study also confirms the fact that, not only polyphenols but there is substantial contribution of nonphenolic compounds also in actually delivering antioxidant and other activities.

ACCT and HECT extracts are also found significantly rich in offering antimitotic and anti-proliferative activity, which can be directly correlated with the presence of higher amount of total flavonoids and polyphenols. The selected plant *Clitoria ternatea* was found beneficial in the preclinical *in vitro* studies on treatment of cancer. The active extracts provide a new insight in the treatment of cancer because of enrichment of active secondary metabolites.

The active extracts shall be subjected to deeper research into various aspects of their anticancer potential and for knowing their precise mechanism of action.

Therefore, in future, the active extracts as well as its active isolated constituents from *C. ternatea* can be a good source of phytomedicines in the management, treatment and cure of different type of cancer. Also, it opens up scope for the synthesis of such active chemical constituents and further evaluation, if there is an increase in the demand from the commerce.

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