Phytochemical study and pharmacological activity of *Terminalia chebula* fruit extracts activity as Dihydrofolate Reductase enzyme inhibitors associated with antioxidant effect: *In vitro* study

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Abstract:

Dihydrofolate reductase (DHFR) is a fundamental enzyme in producing the precursor of purines and pyrimidines for biosynthesis of DNA, RNA and amino acids at various stages. It is considered the key target for both anticancer and antimicrobial drug design.

Terminalia chebula has unique phytoconstituents which are employed broadly in the development of medications against different diseases. It has been established that *Terminalia chebula* fruit could be used as therapeutic agent for cancer treatment. The aim of study was to evaluate the inhibitory effect of *T. chebula* fruit extract against DHFR enzyme activity and assessment the antioxidant and scavenging activity of *T. chebula* fruit extract, using DPPH and reducing activity tests *Terminalia chebula* fruits where extracted. The anti- DHFR enzyme activity was assessed *in vitro* for the four extracts of *Terminalia chebula* fruit and MTX. Phytochemical analysis of screening test, gas chromatography-mass spectrometry (GC-MS) analysis and high-performance liquid chromatography (HPLC) was done for the extract with highest biological activity. Antioxidant and radical scavenging activity of the extract with highest biological activity were evaluated via DPPH [1, 1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β-picrylhydrazyl] and reductive ability test. The percent of DHFR inhibiting activity for the cold methanolic extract was the highest and it was higher than that of MTX (96.0±1.4% vs. 89.0±1.1%, respectively), therefore, it was selected for the proceeding assay. Phytochemical analysis showed that the cold methanolic extract of *T. chebula,* showed a positive reaction for alkaloids, flavonoids, phenolic compounds, steroids and saponins. Besides, GC-MS analysis showed the presence of pyrogallol compound, while HPLC analysis recorded 3 major peaks with different retention times that were semi-identical to gallic acid, rutin and quercetin standard. The highest radical scavenging activity of *T.chebula* cold methanolic extract and ascorbic acid according to DPPH were (80.1±2.04% and 85.83±2.1%, respectively) at the maximum studied concentration (200µg/ml), where the activity of ascorbic acid was significantly higher ($p \leq 0.05$) than that of *T.chebula*. Meanwhile, the reductive ability of the cold extract was significantly higher ($p \leq 0.05$) than that of vitamin E (0.72±0.15 and 0.41±0.08, respectively) at the maximum studied concentration (250µg/ml). These results suggesting the cold extract of *Terminalia chebula* has *in vitro* prominent anti-dihydrofolate reductase activity which is better than that of MTX.

Key words: *Dihydrofolate reductase (DHFR), Terminalia chebula, phytochemical analysis, antioxidant activity.*

دراسة كيميائية وفعالية مستخلصات الميثانول لثمرة اللوز الهندي الدوائيه كمثبط لانزيم الدايهادرو فوليت ريدكتيزو كمضاد للاكسدة: دراسه خارج جسم الكائن الحي

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الخلاصة:

دايهايدرو فوليت ريدكتيز (DHFR)هو انزيم اساسي في انتاج مشتقات البيورينات والبيريميدينات لصناعة الحمض النووي الرايبوزي منقوص الاوكسجين (DNA) والحمض النووي الرايبوزي, (RNA) والاحماض الامينية في مختلف المراحل. ويعتبر الهدف الرئيسي لكلا تصاميم مضادات السرطان والمضادات الحيوية. ثمرة اللوز الهندي(الاهليلج) (Terminalia Chebula)هي نبتة طبية مشهورة تمتلك مكونات نباتية فريدة استخدمت بنطاق واسع في تطوير الادوية ضد مختلف الامراض. الهدف: التقييم المختبري لقدرة مختلف مستخلصات الميثانول لثمرة اللوز الهندي كمثبط لانزيم الدايهادرو فوليت ريدكتيز واختيار المستخلص المثبط الافضل للانزيم وفعالية المستخلص كمضاد للاكسدة.

تم قياس فعالية منع نزيم الدايهايدرو فولايت ريدكتيز مختبريا بواسطة سيكما كت وحسب البروتوكول القياسي المعروف تجاريا والمخصص لهذا الفحص للمستخلصات الاربعه للنبات للحصول على الستخلص الاكثرتثبيطا لفعالية الانزيم.وتم اجراء التحليل الكيميائي النباتي وطيف الكتلة اللونية للغاز بواسطة جهاز كروماتوغرافيا الغاز – مطياف الكتلة لهذا المستخلص المفضل ثم بعد ذلك تم اجراء تحليل مختلف لنفس المستخلص بما في ذلك الكشف عن المكونات النباتية بواسطة تقنية كروماتوغرافيا السائل عالية الدقة. بينما جرى تقييم الفعالية المضادة للاكسدة للمستخلص الميثانولي المفضل مختبريا بواسطة ثنائي الفينيل بكرل هايدرازيل DPPH 1,1وفعاليته في كنس الجذور الحرة وبواسطة فحص قابلية الاختزال للمستخلص . نسبة تثبيط نشاط انزيم دايهايدرو فوليت ريدكتيز بواسطة المستخلص البارد كان اكثر من الميثوتريكسات (96± 1.4%ضد 89± 1.1%, بالتتابع),لذلك تم اختيار المستخلص البارد للدراسة داخل جسم الكائن الحي. كشف الفحص الكيميائي النباتي لمستخلص الكحول البارد لثمرة اللوز الهندي عن وجود مركبات القلويدات والفلافونويدات ومركبات الفينول والستيرويدات والصابون. بينما اظهر تحليل الطيف الكمي للغاز وجود مركب البايروكالول.واظهرت تقنية كروماتوغرافيا السائل عالية الدقة HPLC وجود ثلاث مركبات تمتلك وقت استباق مختلف لكل مركب وهذا الوقت شبه مماثل لوقت المركبات القياسية المستخدمة (حامض الكالك و الروتين والكورسيتين) القياسي. نشاط الكسح الجذري الاعلى للمستخلص الميثانولي البارد وحامض الاسكوربيك وفقا لفحص داي فينيل بكرل هايدرازيل كان (80.1 ± 1.1% و85.83±2.1%,بالتتابع) عند اعلى تركيز مدروس (200مكغم/مل), فعالية حامض الاسكوربيك كانت اكبر من فعاليةالمستخلص .(p≤0.05) وفي نفس الوقت قوة الاختزال للمستخلص البارد كانت اعلى (p≤0.05) من فيتامين E (0,72±0.15و 0.41 ± 0.08, بالتتابع)عند اعلى تركيز مدروس (250مكغم/مل) على الرغم من ذلك, نشاط الكسح الجذري لثنائي داي بكرل هايدرازيل واختبار قدرة الاختزال اعتمدوا على التركيز.

الكلمات المفتاحية: دايهايدرو فوليت ريدكتيز, ثمرة اللوز الهندي, التحليل الكيميائي النباتي ,نشاط مضادات الاكسدة.

Introduction

Dihydrofolate reductase (DHFR) is an important enzyme in producing the precursor of purines and pyrimidine for biosynthesis of RNA, DNA and amino acids at various stages [1]. Nicotinamide adenine dinucleotide phosphate (NADPH) donate electron to dihydrofolate (DHF) by action of DHFR, through a protonation to produce tetrahydrofolate (THF) that required for *de novo* synthesis of purines, thymidylate, as well as some amino acids [2]. Dihydrofolate reductase is a therapeutic target for disease that related to opportunistic infections; autoimmune and metabolic disorders [3] and a key target for anticancer drug design [4].

Many epidemiologic researches discovered that fruits and vegetables consumption decrease chronic illnesses, with fewer adverse reactions. They have elements possess chemoprotective and chemotherapeutic effects that could reduce the opportunity of having malignancy [5].

Several herbs have marked inhibitory effect against DHFR [6] such as: *Sanguinaria canadensis* roots [7], *Caralluma sinaica* also identified as potential anti-neoplastic agent [8]. *Terminalia chebula* (*T.chebula*) (F*: Combretaceae*) called as 'King of Medicine*'* is a famous medicinal plant utilized in Unani System of Medicine (USM) [9]. It has unique phytoconstituents which are employed broadly in the development of medications against different diseases [10]. It contains major active compounds such as phenols, flavonoids and tannins. It is a rich source of gallic acid [11]. The main compounds among flavonoids that are reported from the fruits include rutin and quercetin. *T.chebula* can be used to treat numerous diseases such as conjunctivitis, wound infection, gargling in mouth, diarrhea, vomiting, asthma, gout, ulcers, dysuria, retention of urine and diabetes and exhibits a numerous pharmacological activity such as: antioxidant, hepatoprotective, antidiabetic, antimicrobial, antiarthritic, anti-inflammatory,antimutagenic, cardio-protective and antiproliferative activity [12]. The present study was aimed to evaluate the inhibitory effect of *T. chebula* fruit extract against DHFR enzyme activity and assessment the antioxidant and scavenging activity of *T. chebula* fruit extract, using DPPH and reducing activity tests.

Materials and methods

Chemicals: Methanol 99% from Alpha chemo, India. Deionized water from AL Mansour company/Iraq. Dihydrofolate Reductase Assay Kit from Sigma-Aldrich/ USA. Gallic acid, Quercetin and Rutin standards were purchased from Changdu Biopurify, China. Ascorbic acid from Merck/Germany. DPPH (1,1-diphenyl-2-picrylhydrazyl) from Sigma-Aldrich/ USA.

The plant: The fruits of *Terminalia chebula* were collected in November 2020 from Iraq and then it was identified in the Department of Pharmacognosy/ Collage of Pharmacy/ Mustansiriyah University and the Department of Biology/College of Science/ University of Baghdad by professional plant taxonomist. Then fruits had been stored until extraction process.

Plant extraction: *Terminalia chebula* fruits were carefully cleaned and were air dried at room temperature (20-25°C). Then were grinded to a coarse powder by a hand mortar. They were subjected to two extraction methods. The first method was cold extraction, in which two 100 grams of the plant powder were extracted, the first 100gm was soaked in absolute methanol 99% (1000 ml) and the second 100 gm soaked in aqueous methanol (70% methanol, 30%water) separately for 24 hours at room temperature with stirring from time to time. The second method was hot extraction, in which two 50 grams of the powder were extracted, the first 100gm was macerated in absolute methanol (500 ml) and the 2nd 50gm was macerated in aqueous methanol (70 methanol: 30 water) separately at 65°C for 5 hours using the Soxhlet apparatus. Then the four extracts were dried by rotary evaporator until became concentrated, then was filtered by Whatman filter paper (No. 1), air dried and the yield was weight and labeled (depending on the following equation) then kept in dark glass vials and stored at-20°C until used [13].

Yield of extract = Weight of material obtained /Weight of starting material × 100.

Assessment of Anti-Dihydrofolate Reductase (DHFR) Activity: The assessment of the activity of DHFR enzyme by the four extracts that have been resulted from cold and hot extraction of *Terminalia chebula* fruit was determined by using a commercially available kit from Sigma Aldrich / U.S.A. This work was done in the Educational Laboratory for College of Pharmacy / Mustansiriyah University. This kit is used for the detection of DHFR activity and for screening DHFR inhibitors. Methotrexate (MTX) was also assessed by

this kit and same procedure. [8]. Principle assay of activity of DHFR is based on the ability of DHFR to catalyze the reversible NADPH-dependent reduction of dihydrofolic acid to tetra-hydro-folic acid, NADPH acting as the hydrogen donor and was spectrophotometrically follow the decrease in absorbance at 340 nm.

Dihydrofolic acid +NADPH+H^+ $\xleftrightarrow{\text{DHFR}}$ tetrahydro folic acid+ $NADP^+$

Phytochemical screening: The extract of *Terminalia Chebula* with highest biological activity was subjected to several chemical tests for recognizing different phytoconstituents like flavonoids, alkaloids, polyphenols, saponins and steroids by using standard procedures [14,15].

Gas chromatography-mass spectrometry (GC-MS analysis): This technique commonly used for both qualitative and quantitative analysis of a sample containing a variety of organic compounds [16]. This work was done in the Ministry of Science and Technology, Environment and Water Department. The GC-MS was carried out using a Schimadzu (QP2010) PLUS system. The column used in GC-MS system was (optima-5ms, Medium non polar 30m length, 0.25 um thickness). The chemical compounds from extract with highest biological activity were identified by comparing between the retention times of the peaks obtained chromatographically [17].

High-Performance Liquid Chromatography Analysis (HPLC): The HPLC analysis of the extract with highest biological activity was performed in the Educational Laboratory for College of Pharmacy / Mustansiriyah University. It was carried out by prominence HPLC Shimadzu. The separation was achieved in a reversed phase (RP) C18 column 500(250 mm, 4.6 mm, 5 micro m) at 25 ℃. Detection of gallic acid was carried out at 272 nm using UV detector [18]. While in case of rutin and quercetin detection, it was carried out at 356 nm using UV detector, and then the absorption spectra and retention times of relevant peaks were compared with those of standard compounds [19,20].

Assessment of Antioxidant Activity: Antioxidant activity of the extract with highest biological activity of *Terminalia chebula* that resulted in the DHFR assay test was assessed *in vitro* by determining 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity and reducing power. The procedures were performed in the educational laboratory of the College of Pharmacy / Mustansiriyah University.

a. DPPH [1, 1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β-picrylhydrazyl)] radical scavenging activity: One ml of both plant extract and ascorbic acid (200, 150, 100, 50, 25 and 12.5µg /ml) were added to 4ml of methanol solution of DPPH. The solution of ascorbic acid was used as the standard. The DPPH solution was served as a control. After incubation period at 37°C for 30 minutes, the absorbance was read at 517nm against blank. The scavenging potentials activity of both antioxidants (extract/standard) is detected and measured by the degree of discoloration (purple to yellow) and the change in the absorbance. The procedure was achieved in triplicate. The following equation was used to calculate the inhibition of free radical in percent by using DPPH [21]:

% DPPH radical scavenging activity = $\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}} / \text{Absorbance}_{\text{control}} \times 100$

b. Reductive ability: One ml of the fruit extract of *Terminalia Chebula* and the standard vitamin E at various concentrations (25, 50, 100, 150, 200 and 250 µg /ml was added to 2.5 ml potassium ferricyanide and 2.5 ml phosphate buffer (0.2 M, pH 6.6), then the mixture was incubated for 20 min at 50 C°. After that, the TCA (10%) was added to the mixture and centrifuged at 5000 rpm for 10 minutes. then 2.5 ml from the upper layer was removed and mixed

with 0.5 ml FeCl3 (0.1%) and 2.5 ml DW. The absorbance was measured at 700 nm in the spectrophotometer. All the tests were done in triplicates. A higher absorbance of the reaction mixture indicates a higher reducing power [22].

Statistical analysis: Statistical analysis was performed by using statistical package for the social science (SPSS) (version 24). Normally distributed data of this study was provided in the form of mean ± standard deviation of the mean (M±SD). ANOVA test was used for comparison between more than two groups followed by post-hockey test. Sample t-test was used for comparison between two groups. The results considered significant when the *p*-value was ≤0.05.

Result

Extraction and yield percent of *Terminalia chebula* fruit extract: Four extracts were obtained after extraction of *Terminalia chebula* fruit by the two extraction methods. In general, cold extraction of *Terminalia chebula* fruit gave higher percentage of yield than hot extraction and specifically the aqueous cold methanolic extract had the best yield percentage than other extracts, as shown in the table (1).

Table (1): Yield percent of the four extracts of *Terminalia chebula* fruit obtained after methanolic, aqueous methanolic cold and hot extraction.

Type of extracts	Weight(gram)	Percentage (%)
Cold absolute methanolic extract	32.4	32.4
Hot methanolic extract	15.8	31.6
Aqueous cold methanolic extract	44	44
Aqueous hot methanolic extract	20	40

Anti-dihydrofolate reductase activity: The inhibitory effect of the four extracts of Terminalia chebula fruit as well as methotrexate on dihydrofolate reductase enzyme activity was assessed. The cold methanolic extract (CMETC) resulted the highest inhibitory activity (96%±1.4) than other extracts and MTX, table (2) and figure (1). Cold and aqueous cold extract showed a significant increase in the inhibitory activity versus MTX (p≤0.05). While hot and aqueous hot extract DHFR inhibitory % were significantly lowered compared MTX, therefore, the cold CMETC was the extract selected to be used in the following experimental tests.

Table (2): *In vitro* % of inhibition of Dihydrofolate reductase activity by methanolic extract of *Terminalia chebula* fruit and methotrexate.

Type of extract	% DHFR inhibition± SD
Cold absolute methanolic extract	96.0 ± 1.4^{a}
Hot methanolic extract	85.0 ± 1.4^{b}
Aqueous hot methanolic extract	82.0 ± 2.6^{bc}
Aqueous cold methanolic extract	90.0 ± 1.6^{d}
Methotrexate	89.0 ± 1.1^{de}

Data were expressed as means ± standard deviation. The statistical analysis done by using one way ANOVA followed by post hoc test. Different superscript small letters (a, b, c, d, e) indicate statistically significant difference among groups ($p \leq 0.05$). DHFR: dihydrofolate reductase.

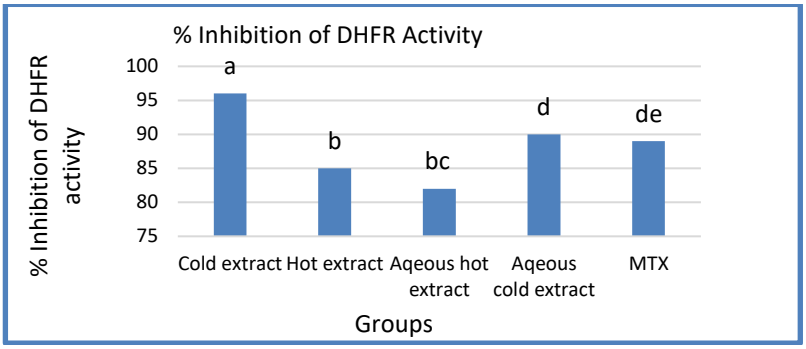


Figure (1): Inhibition % of Dihydrofolate reductase (DHFR) activity by methanolic extract of Terminalia chebula fruit and methotrexate, (P≤0.05) indicate a significant difference. Small letters (a, b, c, d, e) indicate statistically significant difference among groups. DHFR: dihydrofolate reductase, SD: standard deviation. MTX: methotrexate.

Phytochemical screening tests:

Qualitative phytochemical studies were performed on the cold methanolic extract of *T.chebula* fruit (CMETC).The results showed the presence of alkaloids, flavonoids, phenols,saponin and steroids, as shown in table (3)

Table (3): Results of extract preliminary study.

Compounds	Test for identification	Results
Flavonoids	ferric chloride neutral	+
Alkaloids	Dragendroff ʾs test	+
Phenols	ferric chloride	+
Steroids	Liberman-test	+

+: Positive (presence)

Gas chromatography-mass spectrometry (GC-MS) analysis: The most important recognized chemical that showed in GC-MS was pyrogallol. Figure (2):

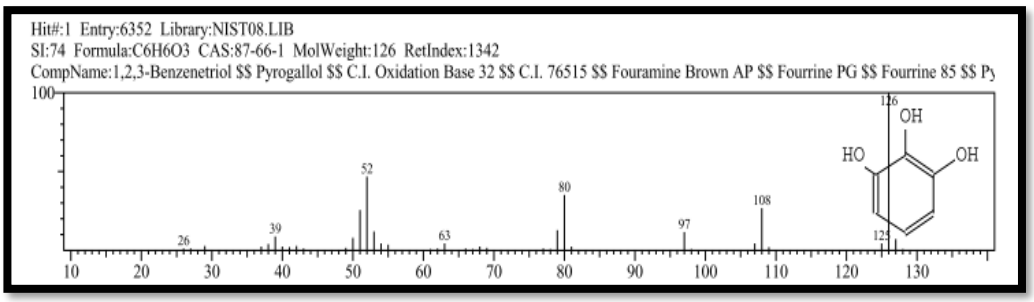
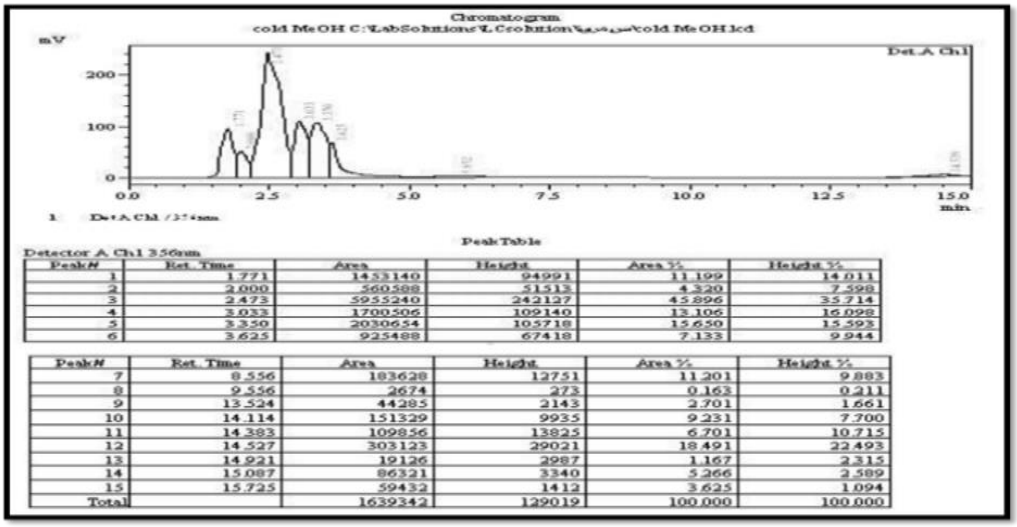


Figure (2): Gas Chromatography-Mass Spectrometry (GC-MS) analysis of cold methanolic extract of *T.chebula* showing pyrogallol structure.

High performance liquid chromatography (HPLC):

Three major peaks having different retention time are resulted in HPLC analysis for CMETC and were semi-identical to rutin, quercetin and gallic acid standard. The peaks that have retention time (3.033 and 15.087minutes) as shown in figures (3) were semi-identical to the peaks of standard rutin and quercetin (3.022 and 15.225

minutes, respectively), as shown in the figure (4). Meanwhile, the peak that has retention time (3.837minutes) in figure (5) has a retention time semi-identical to the peak of standard gallic acid (4.060 minutes), as shown in figure (6).

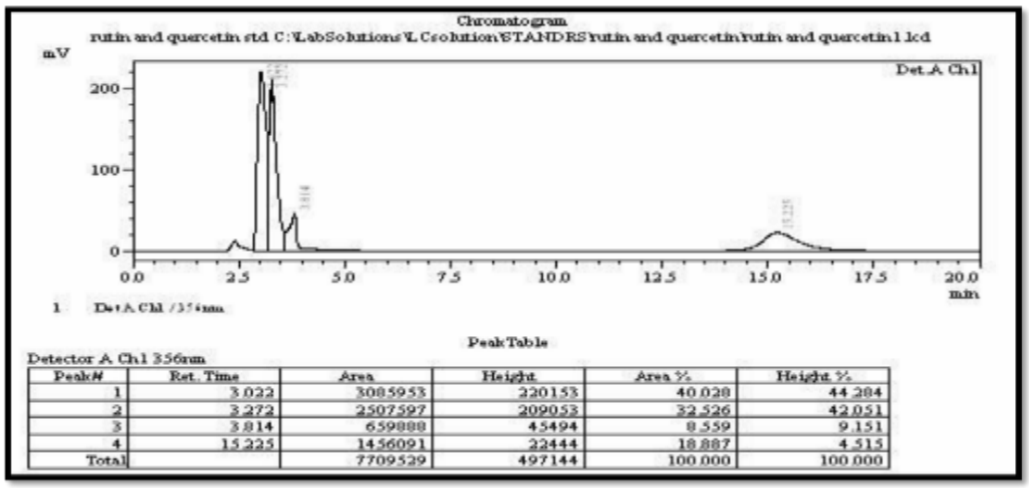


Peak Table

Detector A Ch1 356nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.771	1453140	94991	11.199	14.011
2	2.000	560388	51513	4.320	7.598
3	2.473	5955240	242127	45.896	35.714
4	3.033	1700505	109140	13.106	16.098
5	3.350	2030654	105718	15.650	15.593
6	3.625	925488	67418	7.133	9.944
7	8.556	183628	12751	11.201	9.883
8	9.556	2674	273	0.163	0.211
9	13.524	44285	2143	2.701	1.661
10	14.114	151329	9935	9.231	7.700
11	14.383	109856	13825	6.701	10.715
12	14.527	303123	29021	18.491	22.493
13	14.921	19126	2987	1.167	2.315
14	15.087	86321	3340	5.266	2.589
15	15.725	59432	1412	3.625	1.094
Total		1639342	129019	100.000	100.000

Figure (3): HPLC analysis for cold *Terminalia chebula* fruit extract.

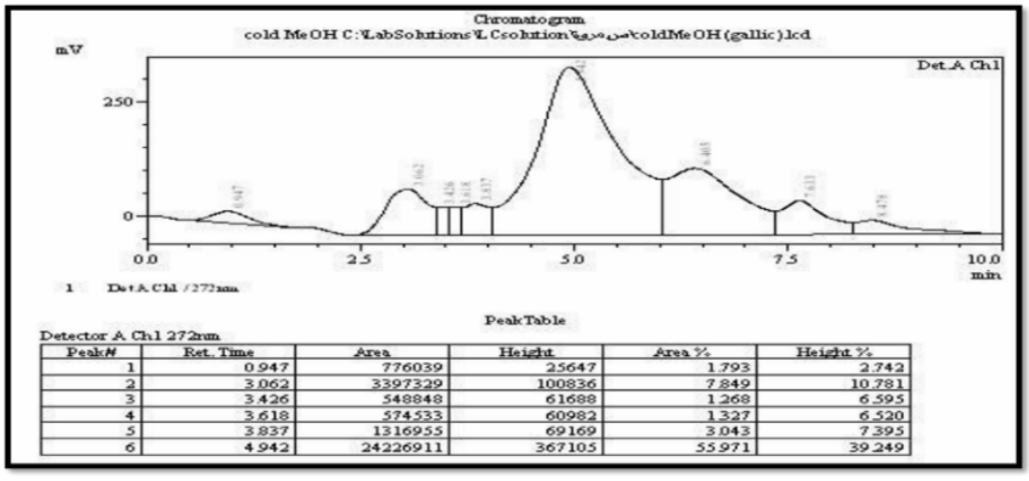


Peak Table

Detector A Ch1 356nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.022	3085953	220153	40.028	44.284
2	3.272	2507597	209053	32.526	42.051
3	3.814	659888	45494	8.559	9.151
4	15.225	1456091	22444	18.887	4.515
Total		7709529	497144	100.000	100.000

Figure (4): HPLC analysis of standard rutin and quercetin



Peak Table

Detector A Ch1 272nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.947	776039	25647	1.793	2.742
2	3.062	3397329	100836	7.849	10.781
3	3.426	548848	61688	1.268	6.595
4	3.618	574533	60982	1.327	6.520
5	3.837	1316955	69169	3.043	7.395
6	4.942	24226911	367105	55.971	39.249

Figure (5): Analysis of the cold fruit extract of *Terminalia chebula* by HPLC.

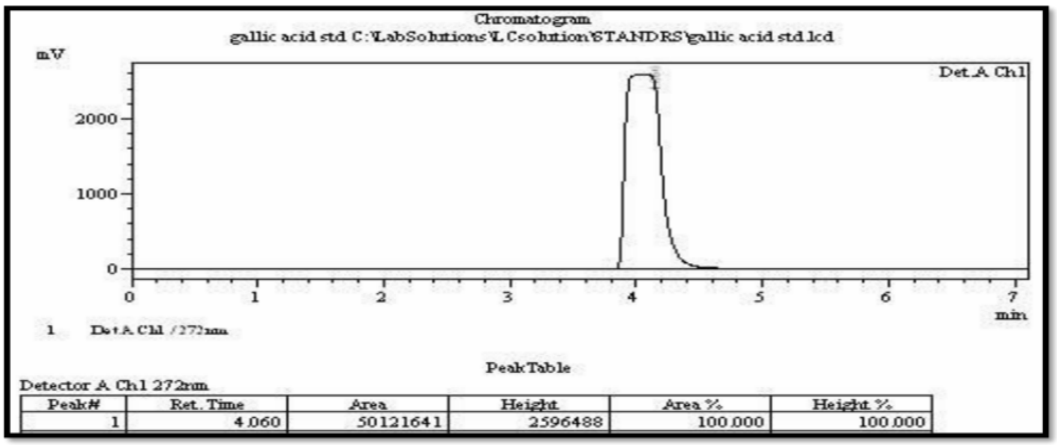


Figure (6): HPLC analysis of Gallic acid standard.

Anti-oxidant activity of methanolic extract for *Terminalia chebula* fruit.

A. DPPH radical scavenging activity: The percentage of DPPH radical scavenging activity of (CMETC) compared with standard ascorbic acid (vitamin C) in this study found that the highest radical scavenging activity of CMETC and ascorbic acid were (80.1±2.04% and 85.83±2.1%, respectively) at the maximum concentration (200µg/ml), where the activity of ascorbic acid was significantly higher than that of CMETC ($p \leq 0.05$), table (4), figure (7). The half maximum inhibitory concentration (IC50) of CMETC was (61.4 µg/ml), (R^2=0.8568), while the IC50 of ascorbic acid was (22.7µg/ml), (R^2=0.8527), as shown in figures (8 A, B).

Table (4): DPPH(1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity percent of cold methanolic extract of *Terminalia chebula* and ascorbic acid.

Concentrati (µg /ml)	%DPPH radical scavenging activity of CMETC extract (M	%DPPH radical scavengi activity of Vitamin C (M±
12.5	28.97±2.21	40.14±1.7*
25	38.78±4.12	49.23±2.55 *
50	51.21±1.17	60.21±3.11*
100	72.0±2.24	78.35±1.85 *
200	80.1 ± 2.04	85.83 ±2.13 *

Data were expressed as means ±SD. The statistical analysis done by using sample t-test. Asterisk (*) represents significant difference between the extract and vitamin c (standard). *P*-value≤0.05 indicate a significant difference. DPPH: diphenyl-picrylhydrazyl, CMETC: cold methanolic extract of *Terminalia chebula.* SD: standard deviation.

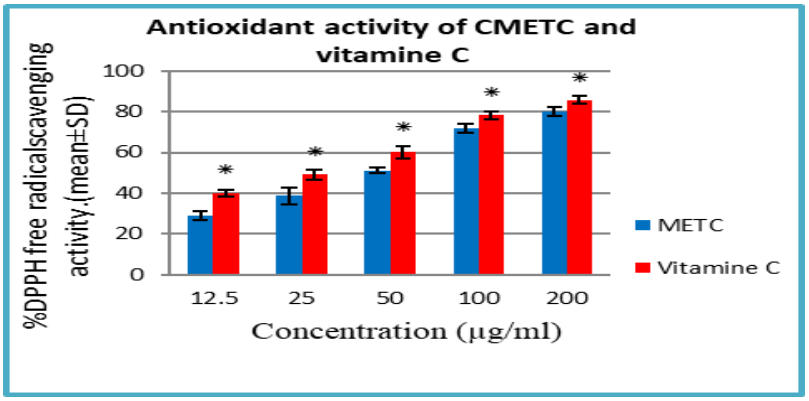
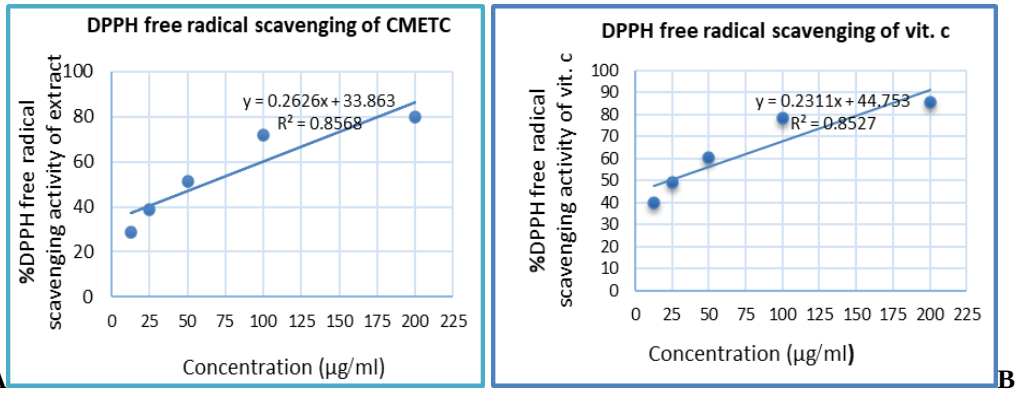


Figure (7): DPPH(1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity of (CMETC) and vitamin C. Asterisk (*) represents significant difference between the extract and vitamin c (standard) (*P*-value≤0.05) indicate a significant difference between the extract and vitamin c (standard). CMETC: cold methanolic extract of *Terminalia chebula* fruit. DPPH: diphenyl-picrylhydrazyl**.**



A B

Figure (8): Dose-response curve A): Cold methanolic extract of Terminalia chebula fruit, B): Vitamin C for radical scavenging activity. CMETC: cold methanolic extract of *Terminalia chebula* fruit. DPPH: diphenyl-picrylhydrazyl

B- Reductive ability

The results revealed that the absorbance of CMETC was significantly higher at all concentrations (25, 50,100,150,200 and 250 µg/ml) when compared with the absorbance of vitamin E (standard) ($p \leq 0.05$), table (5) and figure (9). The IC50 values for the extract and vitamin E were (27.6 µg/ml, R^2 = 0.9443, and 38.3µg/ml, R^2= 0.8928, respectively) and calculated using the linear regression equation from the dose-response curve, figure (10 A, B).

Table (5): Reductive ability of *Terminalia chebula* methanolic extract and vitamin E.

Concentrations (µg/ml)	Reductive ability of CMETC (M± SD)	Reductive ability of (M ±SD)
25	0.43±0.14	0.11±0.08*
50	0.41±0.13	0.21±0.1*
100	0.49±0.12	0.25±0.1*
150	0.52±0.11	0.26±0.06 *
200	0.72±0.15	0.40±0.11 *
250	0.72±0.15	0.41±0.08*

Data were expressed as means ±SD. The statistical analysis done by using sample t-test. Asterisk (*) represents significant difference between the extract and vitamin c (standard). *P*-value≤0.05 indicates a significant difference.

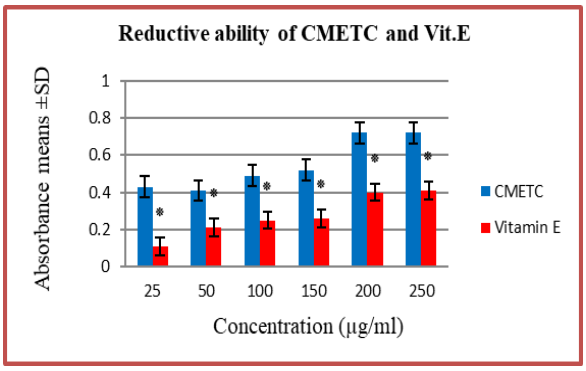
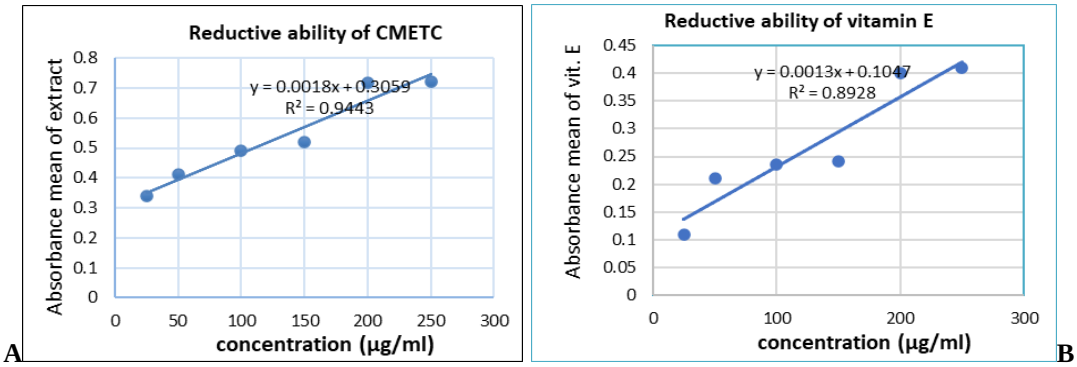


Figure (9): Reductive ability of *Terminalia chebula* cold methanolic extract and vitamin E. The statistical analysis done by using sample t-test. Asterisk (*) represents significant difference between the extract and vitamin c (standard) CMETC: Cold methanolic extract of *Terminalia chebula*.



A B

Figure (10): Dose-response curve for reductive ability A): cold methanolic extract of *Terminalia chebula* fruit, B): vitamin E.

Discussion:

In this study, the highest percentage of yield was 44% for the aqueous methanolic(70%methnol+30%water) cold extract, these results were agreed with Mandeville A *et al* (2018) and Promila P *et al* (2018) study which showed that methanol gave relatively high yields of extracted material than other solvents like ethyl acetate and chloroform [23,24]. Manosroi A *et al* (2010) confirmed that the aqueous methanolic extract has higher yield than methanolic extract [25]. The polarity of water is more than the polarity of methanol, and the polar solvents could extract *T.chebula* at higher yield [26]. *T. chebula* fruit extracts in methanol and water possessed high amount of total phenolic and total tannin content. The

bioactivity of the extract may be weakened with high temperature extraction for the long time by soxholet[27]. Regarding to anti-DHFR activity in this study, it was found that the cold methanolic extract possess the most potent inhibitory effects on the enzymatic activity of DHFR (96%) and was significantly higher than that of known inhibitor MTX which was 89%. Therefore, CMETC appeared to have a potential to be used as DHFR antagonist and might be used as anticancer agent alone or in combination with MTX to decrease the adverse drug reaction of this cytotoxic agent. This may be due to the presence of polyphenol compounds such as pyrogallol that showed in GC-MS. There is agreement between this study and Baharuddin *et al* (2015) study which reported the inhibitory effect of *Qureous infectoria* on DHFR because these two plants shared the phytochemical pyrogallol phenolic compound that has three hydroxyl groups on the same benzene ring which allows their oxidation or autoxidation to act as ROS scavengers [(28)]. The presence of gallic acid (GA) and flavonoid such as quercetin and rutin which were detected in CMETC by HPLC test also suggested having a role in inhibition of DHFR.

In the presented study, the preliminary screening for cold *Terminalia chebula* fruit extract demonstrated that all the tests were positive. This result was agreed with (Zeeshan U *et al* (2018)) study Swargiary A *et al* (2019) study which were showed that the cold methanolic extract of *T. chebula* showed that the cold methanolic extract of *T. chebula* contain high level of phenolics, saponins, flavonoids, alkaloids and tannins [29,30]. Gas Chromatography-Mass Spectrometry (GC-MS) analysis in the present study, showed the presence of pyrogallol (1, 2, 3-benzenetriol) with similarity index (SI) of 76%. It is a phenolic compound and one of hydrolysable tannins which might result from degradation of gallic acid and can be used as antioxidant [31] and anti-DHFR as mentioned above. The HPLC analysis of the methanolic extract of *T. chebula* in this study showed three prominent peaks that have been identified to be rutin, quercetin and gallic acid, respectively using standard solutions under similar condition. The peaks of the extract were semi-identical to the peaks of the standard. This was in lined with Sabir SM (2020) and Naik GH *et al* (2004) studies which revealed the presence of quercetin, rutin and gallic acid in *Terminalia chebula* fruit extract by HPLC analysis [32,33].

Regarding the antioxidant activity in this study, the cold extract showed considerable antioxidant potential *in vitro* study. It was found that the CMETC in all concentrations tested showed considerable activity in reducing the DPPH radical although not better than vitamin C at the same concentration, table (4 and 5) and figure (7). The half maximal inhibitory concentration (IC50) value of *T. chebula* fruit extract was (64.1 µg/ml) and it reflects its effective scavenging ability while the IC50 value of vitamin C was (22.7µg/ml). This finding was in lined with **Rani AA *et al* (2016)** study, which showed that the reducing capacity of *T. chebula* fruit extract, although not better than the standard, but showed considerable activity [(34)]. This effect could be attributed to that the extract of *T.chebula* fruit has the ability to donate proton and can served as free radical scavenger or inhibitor. Therefore, in this study, the antioxidant activity of *T.chebula* fruit extract might be associated with the presence of phenolic (OH) compound.

The reducing power assay was also used in this study to measure the scavenging power of CMETC. The reducing power measured the extract ability to convert Fe^{+3} to Fe^{+2} at multiple concentrations and compared with vitamin E as standard. It was seen that CMETC possess better antioxidant activity and was significantly higher than the standard vitamin E, and relation was concentration-dependent with maximum reductive effect at concentration 250 µg

/ml. These results were in lined with Kathirvel A *et al* (2012), who showed that the efficiency of *T.chebula* extract by methanol solvent exhibit better reducing power than the standard vitamin E and their ability to reduce $Fe^{+3}$to Fe^{+2} due to the presence of phenolic compounds that might have the ability to donate their hydrogen atoms and neutralize the free radicals by their reducing property to prevent the onset or propagation of oxidative diseases[35].

Conclusion:

Methanolic extraction of *T. chebula* fruit give a good percent of yield according to the method of extraction used. Cold extract of *T. chebula* fruit extract is a rich source of chemical constituents (flavonoids, phenolic compounds, and others) that have *in vitro* potent antioxidant properties, and prominent anti-dihydrofolate reductase activity which is comparable to that of MTX, or even better. Cold extract of *T.chebula* also has a good antioxidant activity compared vitamin C and vitamin E.

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