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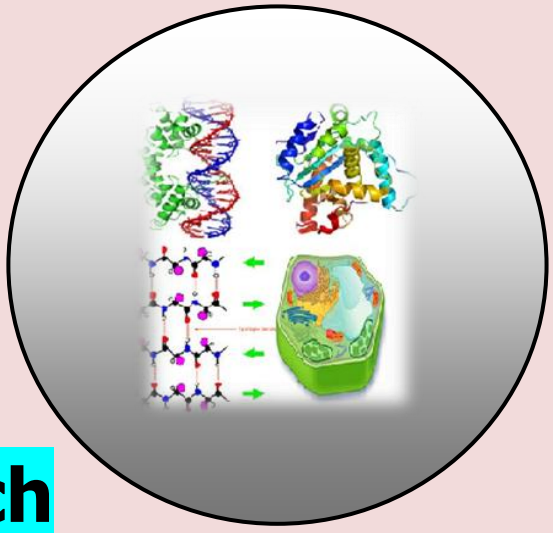
ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

UGC Approved Journal No. 62923
MCI Validated Journal
Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66

J. Biol. Chem. Research
Volume 36 (1) 2019 Pages No. 151-156



Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited in various International and National Scientific Databases

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 36, No. 1: 151-156, 2019
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 36/01/88/2019
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ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

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RESEARCH PAPER

Received: 25/12/2018 **Revised: 23/01/2019** **Accepted: 24/01/2019**

In vitro Anti-oxidant Activity of Various Extracts of *Terminalia catappa* leaves and *Terminalia chebula* fruits

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ABSTRACT

Objective: The present investigation evaluates the in vitro anti-oxidant activity of various extracts of Terminalia catappa leaves and Terminalia chebula fruits. Material and Methods: The various extracts are prepared by using the soxhlet apparatus. Then, it was subjected to the various in vitro anti-oxidant using DPPH assay, nitric oxide assay, reducing power assay, hydrogen peroxide scavenging activity and superoxide anion scavenging activity. Results: The results of anti-oxidant activity of T. catappa leaves and T. chebula fruits of various extracts have an activity in an in vitro model in a dose-dependent manner. Conclusion: Among the various extracts the ethanolic extract has a higher activity in the both plant extracts. Therefore, further in vivo studies and compound identification studies are warranted.
Keywords: In vitro assay, Anti-oxidant activity, Terminalia catappa and Terminalia chebula.

INTRODUCTION

Free radicals and other reactive oxygen species (ROS) are the important causative factors for the development of number of diseases such as aging, cancer, cardiovascular disease etc., (Eastwood, 1999). Anti-oxidants are the substances, which is present in our body at a low concentration. These substances protect our body against the various type of oxidative damages like cancer, arthritis, diabetes, ageing process etc. The phytoconstituents present in the plants plays a vital role in balancing anti-oxidant mechanism. Numerous chemicals are produced by the plants in different concentrations and the relation between these phytochemicals, which helps the plants to fight against various diseases and insects. Due to the highly expensive and side effects, the researchers are trying to find the drugs from the plant sources without any toxic effect. The various phytoconstituents are present in the plants, the compounds have various biological activities. The numerous plants and their parts are used for the medicinal purposes from ancient times. In now-a-days the researchers are trying to find the mechanism of action of the phytoconstituents and developed the new drugs, because these are inexpensive and have no side effects (Hannah *et al*., 2010). In the present study, focus to find the anti-oxidative property of *Terminalia catappa* leaves and *Terminalia chebulla* fruits by an *in vitro* models.

MATERIAL AND METHODS

DPPH radical scavenging activity (Shimada *et al*., 1992)

To a methanolic solution of DPPH (90.25 mM), an equal volume of ethanol, chloroform and aqueous leaf extract of *T. catappa* and fruit extract of *T. chebula* (25 µg, 50 µg, 100 µg, 150 µg, 200 µg and 250 µg) was added and made up to 1.0 mL with methanolic DPPH. An equal amount of methanol was added to the control. After 20 minutes, the absorbance was recorded at 517 nm in a Systronics UV-visible Spectrophotometer. Ascorbic acid was used as standard for comparison. The inhibition of free radicals by DPPH in percentage terms (%) was calculated by using the following equation, where A control is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound.

$$\% \text{ Scavenging} = \frac{\text{A Control OD} - \text{A Sample}}{\text{A Blank}} \text{ X } 100$$

Scavenging activity of nitrous oxide (Garrat, 1964)

A volume of 2 ml of 10 mM sodium nitroprusside prepared in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract, at various concentrations (25 µg, 50 µg, 100 µg, 150 µg, 200 µg and 250 µg). The mixture was incubated at 25°C. After 150 minutes, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent. The mixture was incubated at room temperature for 30 minutes, followed by the measurement of absorbance at 540 nm. The amount of NO radical inhibited by the extract was calculated using the following equation:

$$\% \text{ Scavenging} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \text{ X } 100$$

Reducing power assay (Oyaizu, 1986)

Varying concentrations of ethanol, chloroform, aqueous leaf extract of *T. catappa* and fruit extract of *T. chebula* (25 µg, 50 µg, 100 µg, 150 µg, 200 µg and 250 µg) in double distilled water was mixed with 2.5 mL of phosphate buffer and 2.5 mL of KCN. The mixture was incubated at 50ºC for 20 minutes after which, 1.5 mL of TCA was added and centrifuged at 3000 x g for 10 minutes. From all the tubes, 0.5 mL of supernatant was mixed with 1 mL of distilled water and 0.5 mL of $FeCl_3$. The absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated increasing reducing power. Incubation with water in place of additives was used as the blank.

$$\% \text{ Scavenging} = \frac{\text{A Control OD} - \text{A Sample}}{\text{A Blank}} \text{ X } 100$$

Scavenging of hydrogen peroxide (Ruch *et al.,* 1989)

A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). H_2O_2 concentration was determined spectrophotometrically from absorption at 230 nm in a spectrophotometer (SL 159, UV- Visible Spec, and Elico, India). Extracts (25 µg, 50 µg, 100 µg, 150 µg, 200 µg and 250 µg) in distilled water were added to a H_2O_2 solution (0.6 mL, 40 mM). Absorbance of H_2O_2 at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 of ethanol, chloroform, and aqueous leaf extracts of *T. catappa* and fruit extract of *T. chebula* standard was calculated using the following equation:

$$\% \text{ Scavenging} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \text{ X } 100$$

Superoxide anion scavenging activity (Nishimiki *et al.,* 1972)

1 mL of NBT, 1 mL of NADH solution and varying volumes of all the extracts ethanol, chloroform, aqueous leaf extract of *T. catappa* and fruit extract of *T. chebula* (25 µg, 50 µg, 100 µg, 150 µg, 200 µg and 250 µg) were mixed well. The reaction was started by the addition of 100 µM of PMS. The reaction mixture was incubated at 30° C for 15 minutes. The absorbance was measured at 560 nm in a spectrophotometer. Incubation without ethanol, chloroform, aqueous extract of *T. catappa* leaves and T*. chebula* fruit was used as blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging. The % of inhibition was calculated as shown below:

$$\% \text{ Scavenging} = \frac{\text{A Control OD} - \text{A Sample}}{\text{A Blank}} \text{ X } 100$$

In the present study, the different concentrations of the different extract of (ethanol, chloroform and aqueous) *T. catappa* leaves and *T. chebula* fruits anti-oxidant potential were assessed by *in vitro* models *viz*. DPPH, reducing power assay, NO, superoxide radical and H_2O_2. Ascorbic acid, BHT and α-tocopherol were used as a standard for these assay.

RESULT AND DISCUSSION

DPPH RADICAL SCAVENGING ACTIVITY

Table 1 shows the anti-oxidant potential of *T. catappa* leaves and *T. chebula* fruits on DPPH radical. Highest percentage of inhibition was observed in higher concentrations of the different extracts in both of the plants. The percentage of inhibition was found to be 88.40%, 87.43% and 86.20% in 250 µg/ml of *T. catappa* leaves extracts of ethanol, aqueous and chloroform respectively. The percentage of inhibition were found to be 89.41%, 88.9% and 87.02% of inhibition was observed in 250 µg/ml of *T. chebula* fruits extracts of ethanol, aqueous and chloroform respectively. Similarly, the lowest percentage of inhibition 21.03%, 19.45% and 18.18% were observed in *T. catappa* leaves and 20.32%, 19.12% and 18.75% were observed in *T. chebula* fruits in lower concentrations (25 µg/ml) of different extracts. The higher inhibitory activity, which is similar to the standard ascorbic acid treated values. The IC_{50} values of *T. catappa* leaves are 92.5 µg/ml, 97.5 µg/ml, 100 µg/ml and the *T. chebula* fruits have 90 µg/ml, 92.5 µg/ml and 92.5 µg/ml of IC_{50} values of the ethanolic, aqueous and chloroform extracts respectively. The IC_{50} values standard ascorbic acid is 77.5 µg/ml.The inhibition of DPPH is an important method to find the anti-oxidant potential of plant extracts (Mothana *et al*., 2010). The anti-oxidants donating the hydrogen radical to synthetic long-lived nitrogen radical compound DPPH. The extracts donate the free electrons to the DPPH and it loses its colour. The intensity of colour change is directly proportional to the anti-oxidant potential. In the present study, leaves of the *T. catappa* and *T. chebula* fruits have an anti-oxidant potentially in all the extracts. Among theses the activity is higher in the order of ethanolic, aqueous and chloroform extract against the DPPH. The similar effect is noted in the *Psidium guajava* leaves (Vijayakumar *et al*., 2015).

REDUCING POWER ASSAY

The reductive potential of *T. catappa* leaves and *T. chebula* fruits with standard was shown in Table 2. The reduction percentage was increased in increasing concentration and decreased in decreasing concentration of both of the plants extracts and also in standard α-tocopherol. Different extracts of the *T. catappa* leaves shows 84.13%, 82.06% and 81.16% of inhibitions at 250 µg/ml and 31.01%, 29.15% and 26.21% of inhibitions at 25 µg/ml. Different extracts of *T. chebula* fruits shows 82.21%, 81.11% and 79.17% of inhibition at 250 µg/ml and 29.31%, 27.22% and 26.13% of inhibition at 25 µg/ml. The IC_{50} values of *T. catappa* leaves are 92.5 µg/ml, 100 µg/ml and 137.5 µg/ml and the *T. chebula* fruits are 130 µg/ml, 95 µg/ml and 97.5 µg/ml in the ethanolic, aqueous and chloroform extracts respectively. The IC_{50} value of α-tocopherol is 100 µg/ml. The anti-oxidant compound reduces the ferric ion into ferrous ion, this effect reduces the free radical generations as well as the reluctant, which break the free radical chains. This reducing power of the plant extracts mainly depends on the presence of phenolic content (Divya and Vijaya Anand, 2015). In the present study, the leaves and fruit extracts of all the solvents have a reducing potential. Among these the ethanolic extracts have a high ability of the both of the *T. catappa* leaves and *T. chebula* fruits extracts.

NITRIC OXIDE ASSAY

Table 3 the NO scavenging effect of *T. catappa* leaves and *T. chebula* fruits with standard ascorbic acid. The maximum dose of the both plants extracts were highest percentage of scavenging activity. The lower doses of the both plants extract were lower percentage of scavenging activity. *T. catappa* leaves shows the inhibition at 250 µg/ml were 88.17%, 86.17% and 84.21% and the inhibition at 25 µg/ml were 20.10%, 19.01% and 17.16%. Similarly, *T. chebula* fruits show the inhibition at 250 µg/ml were 89.01%, 87.60% and 86.13% and the inhibition at 25 µg/ml were 19.86%, 17.11% and 16.60%. The standard drug of ascorbic acid, which shows the inhibition of 94.02% of inhibition at 250 µg/ml and IC_{50} is 115 µg/ml. The IC_{50} values of *T. catappa* leaves are 92.5 µg/ml, 100 µg/ml and 122.5 µg/ml and the *T. chebula* fruits are 125 µg/ml, 110 µg/ml and 112.5 µg/ml in the ethanolic, aqueous and chloroform extracts respectively. NO is a reactive radical, it easily reacts with superoxide anion, to produce a stronger oxidant of peroxynitrite, it involves the damage of DNA and nitration of proteins and nucleic acids. The inhibition of NO accumulation is an important therapeutic strategy of natural antioxidants. Divya and Vijaya Anand (2015) prove the NO inhibition activity of *T. catappa* leaves which favours the anti-oxidant nature in an *in vitro* model. This may prove in this study also. In the present study, the *T. catappa* leaves extract have an anti-oxidant activities among the various extracts ethanol have a high activity. *T. chebula* fruits also have an anti-oxidant activity in all the extracts. Here also the ethanolic extracts have a high activity.

SUPER OXIDE RADICAL ASSAY

Table 4 shows the superoxide radical scavenging effect of *T. catappa* leaves and *T. chebula* fruits with standard BHT. The maximum dose of the both plants extracts were highest percentage of scavenging activity.

Table 1. DPPH radical scavenging activity of *T. catappa* leaves and *T. chebula* fruits extract.

S. NO	Concentration of plant extract and standard (µg/ml)	*T. catappa*			*T. chebula*			% of inhibition of ascorbic acid
		% of inhibition of ethanol extract	% of inhibition of aqueous extract	% of inhibition of chloroform extract	% of inhibition of ethanol extract	% of inhibition of aqueous extract	% of inhibition of chloroform extract	
1.	25	21.03	19.45	18.18	20.32	19.12	18.75	27.18
2.	50	40.19	37.15	35.37	42.61	41.30	41.56	48.34
3.	100	54.05	52.20	50.03	56.10	55.05	55.21	65.78
4.	150	65.12	63.40	61.51	63.23	61.03	60.65	72.28
5.	200	72.12	70.14	68.12	73.30	72.12	70.20	80.11
6.	250	88.40	87.43	86.20	89.41	88.9	87.02	92.29
IC_{50}		**92.5**	**97.5**	**100**	**90**	**92.5**	**92.5**	**77.5**

Table 2. Reducing power assay of *T. catappa* leaves and *T. chebula* fruits extract.

S. NO.	Concentration of plant extract and standard (µg/ml)	*T. catappa*			*T. chebula*			% of inhibition of α-tocopherol
		% of inhibition of ethanol extract	% of inhibition of aqueous extract	% of inhibition of chloroform extract	% of inhibition of ethanol extract	% of inhibition of aqueous extract	% of inhibition of chloroform extract	
1.	25	31.01	29.15	26.21	29.31	27.22	26.13	34.76
2.	50	43.32	41.14	39.11	44.10	43.01	41.16	50.12
3.	100	50.30	48.02	46.05	52.11	51.21	50.16	58.56
4.	150	58.21	55.21	53.22	55.50	53.61	51.07	65.43
5.	200	66.04	64.12	63.08	68.11	66.23	64.18	74.23
6.	250	84.13	82.06	81.16	82.21	81.11	79.17	91.11
IC_{50}		**92.5**	**100**	**137.5**	**130**	**95**	**97.5**	**100**

Table 3. Nitric oxide assay of radical scavenging activity of *T. catappa leaves and T. chebula* fruits extract.

S. NO.	Concentration of plant extract and standard (µg/ml)	*T. catappa*			*T. chebula*			% of inhibition of ascorbic acid
		% of inhibition of ethanol extract	% of inhibition of aqueous	% of inhibition of chlorofor	% of inhibition of ethanol	% of inhibition of aqueous	% of inhibition of chloroform extract	
1.	25	20.10	19.01	17.16	19.86	17.11	16.60	21.56
2.	50	45.16	43.10	42.06	48.20	47.55	46.30	46.27
3.	100	50.05	48.70	47.21	49.11	47.21	45.14	51.78
4.	150	64.20	62.19	60.30	68.21	67.09	66.19	75.12
5.	200	75.31	72.10	70.18	73.16	71.11	70.17	80.45
6.	250	88.17	86.17	84.21	89.01	87.60	86.13	94.02
IC_{50}		**92.5**	**100**	**122.5**	**125**	**110**	**112.5**	**115**

The lower doses of the both plants extract were lower percentage of scavenging activity. Ethanolic, aqueous and chloroform extracts of *T. catappa* leaves shows the inhibition at 250 µg/ml were 88.09%, 85.26% and 83.12% and the inhibition at 25 µg/ml were 18.11%, 17.10% and 15.31% respectively. Similarly, ethanolic, chloroform and aqueous extracts *T. chebula* fruits shows the inhibition at 250 µg/ml were 92.14%, 92.17% and 90.01% and the inhibition at 25 µg/ml were 18.01%, 17.12% and 14.02% respectively.

Table 4. Superoxide radical scavenging activity of *T. catappa* leaves and *T. chebula* fruits extract.

S. NO.	Concentration of plant extract and standard (µg/ml)	*T. catappa*			*T. chebula*			% of inhibition of BHT
		% of inhibition of ethanol extract	% of inhibition of aqueous extract	% of inhibition of chloroform extract	% of inhibition of ethanol extract	% of inhibition of aqueous extract	% of inhibition of chloroform extract	
1.	25	18.11	17.10	15.31	18.01	17.12	14.02	19.23
2.	50	33.40	31.21	30.10	34.19	32.17	30.15	40.20
3.	100	47.10	45.15	43.30	50.41	48.20	46.09	56.14
4.	150	66.51	64.21	62.11	69.16	66.01	64.21	72.07
5.	200	79.05	77.13	75.30	81.77	78.23	76.17	86.34
6.	250	88.09	85.26	83.12	92.14	92.17	90.01	93.10
IC_{50}		**92.5**	**112.5**	**117.5**	**120**	**100**	**115**	**117.5**

Table 5. Hydrogen peroxide radical scavenging activity of *T. catappa* leaves and *T. chebula* fruits extract.

S. NO.	Concentration of plant extract and standard (µg/ml)	*T. catappa*			*T. chebula*			% of inhibition of α-tocopherol
		% of inhibition of ethanol extract	% of inhibition of aqueous extract	% of inhibition of chloroform extract	% of inhibition of ethanol extract	% of inhibition of aqueous extract	% of inhibition of chloroform extract	
1.	25	17.12	16.10	15.11	18.10	16.15	14.18	19.10
2.	50	34.40	32.14	31.17	34.11	32.12	31.10	36.13
3.	100	46.16	41.10	42.21	53.01	47.20	45.11	54.05
4.	150	62.15	58.13	63.12	68.15	69.19	63.21	72.10
5.	200	78.13	70.11	71.15	85.18	79.12	75.14	85.19
6.	250	85.11	81.02	80.11	90.11	90.13	88.01	90.12
IC_{50}		**92.5**	**122.5**	**130**	**120**	**95**	**110**	**120**

In the present study, both the *T. catappa* leaves and *T. chebula* fruits extracts have an effective inhibiting of SOD. This may proves the anti-oxidant ability of the *T. catappa* leaves and *T. chebula* fruits in an *in vitro* model among these the ethanolic extracts of the *T. catappa* leaves and *T. chebula* fruits have a similar effect compared to the standard BHT. The IC_{50} value of standard is 117.5 µg/ml and the IC_{50} values of *T. catappa* leaves is 92.5 µg/ml, 112.5 µg/ml and 117.5 µg/ml and *T. chebula* fruits is 120 µg/ml, 100 µg/ml, 115 µg/ml in the ethanolic, aqueous and chloroform extracts respectively.

The enzymatic antioxidants are involved in the direct elimination of ROS (Halliwell and Gutteridge, 1990). SOD is an important anti-oxidant enzyme, it catalyzes the dismutation process. SOD plays a defensive role against the potential oxygen toxicity. It protects the cells from oxidative stress. The percentage of inhibition of this enzyme is directly proportional to the anti-oxidant ability of the plant extracts.

HYDROGEN PEROXIDE ASSAY

Table 5 shows the H_2O_2 radical scavenging effect of *T. catappa* leaves and *T. chebula* fruits with standard α-tocopherol. The maximum dose of the both plants extracts were highest percentage of scavenging activity. The lower doses of the both plants extract were lower percentage of scavenging activity. Ethanolic, aqueous and chloroform extracts of *T. catappa* leaves shows the inhibition at 250 µg/ml were 85.11%, 81.02% and 80.11% and the inhibition at 25 µg/ml were 17.12%, 16.10% and 15.11% respectively.

Similarly, ethanolic, aqueous and chloroform extracts *T. chebula* fruits shows the inhibition at 250 µg/ml were 90.11%, 90.13% and 88.01% and the inhibition at 25 µg/ml were 18.10%, 16.15% and 14.18% respectively. The IC_{50} value *T. catappa* leaves extract on H_2O_2 radical is 92.5 µg/ml**,** 122.5 µg/ml, 130 µg/ml and the *T. chebula* fruits extract is 120 µg/ml, 95 µg/ml and 110 µg/ml in the ethanolic, aqueous and chloroform extracts respectively. The IC_{50} value of standard drug α-tocopherol is 120 µg/ml. The result of H_2O_2 scavenging activity of this study is similar to the results of the *in vitro* anti-oxidant activity of *Cressa cretica* by Priyanka *et al*. (2015). H_2O_2 is a substance, it undergoes some reactions and it produces the hydroxyl radicals in the cells, it may damages the cell membranes. Hence, the inhibition of H_2O_2 is an important work for the antioxidants. These antioxidants donate the electrons to H_2O_2 and prevent the hydroxyl radical formation. In the present study, the extract of *T. catappa* leaves and *T. chebula* fruits potentially inhibits the formation of hydroxyl radicals from H_2O_2. Among the three extracts the ethanolic extracts have a high activity in both the *T. catappa* leaves and *T. chebula* fruits.

CONCLUSION

The present study proved that the extract of *T. catappa* leaves and *T. chebula* fruits have the anti-oxidant activity in the extracts of ethanol, chloroform and aqueous in an *in vitro* model. Among these three extracts ethanolic extract exhibit higher activity. Hence, further *in vivo* studies and compound isolation studies are needed.

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