SECONDARY METABOLITES FROM TERMINALIA CATAPPA AND EVALUATION OF ITS BIOACTIVITIES

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ABSTRACT

Bangladesh is a land of medicinal plants. *Terminalia catappa* is an important medicinal plant of Bangladesh which is used for various pharmacological and biological activities like hepatoprotective, antioxidant, anti-fertility, antidiabetic, anticoagulant and antimicrobial. The coarsely powder dried stem bark sample of *T. catappa* was extracted by the solvent $CHCl_3$. The concentrated extract was then fractionated by flash column chromatography using petroleum ether, ethyl acetate and methanol as solvents respectively. The column fraction was further fractionated with void liquid chromatography. Repeated preparative thin layer chromatography of selective fractions resulted in the isolation of two compounds which were identified as a triterpenoid, taraxerol (**1**) and a steroid, Stigmastane-3, 6-diol (**2**). The structure of these compounds were elucidated by ^{1}H NMR spectroscopy and by comparison with reported values. This is the first report of the isolation of these compounds from this plant. The different extracts also showed significant antibacterial activity against gram positive and gram negative bacteria. The methanol extract did not show significant activity. The antifungal activity of different extract of *T. catappa* demonstrated promising zone of inhibition except *Saccharomyces cerevecae*. In the brine shrimp lethality bioassay, the extract of *T. catappa* exhibited a significant cytotoxic activity. The LC_{50} value of $CHCl_3$ soluble extract was found to be 1.55 µg/ml. *T. catappa* was also reported to have free radical scavenging activity with IC_{50} value of 255.0 µg/ml. Since this plant demonstrated a number of promising pharmacological biological activities, so it can be a good source of natural medicine.

KEYWORDS: *Terminalia catappa,* chromatography, taraxerol, stigmastane-3,6-diol, antibacterial, cytotoxic, antioxidant.

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Introduction

Medicinal plants are those which have been utilized throughout human history for the treatment of different diseases. Medicinal plants also show therapeutic properties or exert beneficial pharmacological effect on the animal body. *Terminalia catappa* is widely distributed throughout the tropical regions of the world with hepatoprotective, antioxidant, anti-fertility, antidiabetic, anticoagulant and antimicrobial activities. The leaves of *T. catappa* contain several flavonoids (ex: kaempferol), several tannins (ex: punicalin), saponines and phytosterols[1].Triterpenic acids were responsible for the anti-inflammatory activity of *T. catappa* leaves[2]. *T. catappa* has shown potent antidiabetic activity[3]. Different extracts of *T. catappa* exhibited potential antioxidant activities[4,5]. The anti-inflammatory activity of *T. catappa* was also reported by several studies [6,7]. A chemical examination of the leaves of *T. catappa* resulted in the isolation of four new hydrolyzable tannins and eight known tannins[8]. Crude extract and the phenolics of *T. catappa* fruits has been found to show inhibition against tumor cell growth. A significant antidiabetic activity against experimental rats has also been reported [9,10]. *T. catappa* also demonstrated anticlastogenic both *in vitro* and *in vivo* studies[11]. Despite the importance of *T. catappa* as a medicinal plant, so far, no systematic analysis has been done yet on this plant. The main purpose of this study was isolation, purification and identification of the compounds from the stem bark extract of *T. catappa* and also evaluates on their antibacterial, antifungal, cytotoxic, antioxidant, analgesic and anti-inflammatory activities. We identified two unique bioactive compounds from *T. catappa*, one triterpenoid, Taraxerol (**1**) and one steroid, Stigmastane-3,6-diol (**2**). The different extracts from *T. catappa* also showed promising antibacterial, antifungal, cytotoxic and antioxidant *activities.*

Materials and Methods

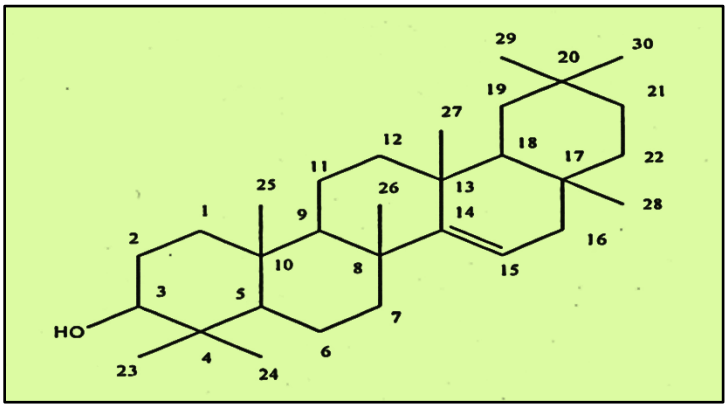
Collection of plant materials (stem barks) from *T. catappa*, dried up and grounded, extraction by appropriate solvents, extracts was fractionated by flash column chromatography, detection of compounds by various spreading reagents and by the use of UV light on the thin layer chromatographic plates. Then isolation of compounds was done by preparative thin layer chromatography and identification of them by the use of

IR and ^{1}H NMR spectroscopy as well as by comparing with reported values.

Then the biological activities have been observed. The biological activities were antibacterial, antifungal, cytotoxic, antioxidant etc. The antimicrobial activity will be observed by disc diffusion method[12], cytotoxic activity was tested by brine shrimp lethality[13,14,] and antioxidant activity was followed by DPPH assay[15]. The biological activities of the different plant extracts as well as pure isolated compounds from them will be studied. The MIC, LC_{50} and IC_{50} values will also be determined.

Results and Discussion

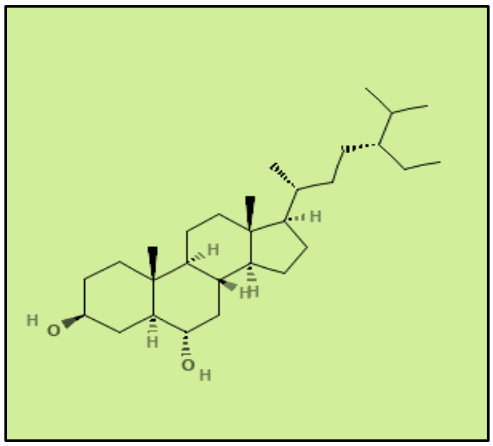
Compound TC-1 was spotted as a single spot with vanillin-H_2SO_4 reagent followed by heating at 110°C for 5 minutes on the silica gel plate. It was obtained as crystals from the fraction no 3 of $CHCl_3$ extract of *T.catappa*. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of TC-1 exhibited eight three proton singles at δ_H 0.78 (H3-24), 0.86 (H3-28), 0.92 (H3-27), 0.98 (H3-25), 1.02 (H3-29), 1.14 (H3-23) and 1.18 (H3-26) which suggested the presence of eight methyl groups in a triterpene. It also revealed a multiplate centered at ∂_H 5.53 which could be assigned for an olefinic proton for H-15. The broad doublet (J=9.2 Hz) at δ_H 3.20 can be demonstrated for a proton at C-3 of a triterpene. The above spectral features were in close agreement to the triterpenoid taraxerol (1) which was confirmed by comparing reported spectral values[16]. Taraxerol is a crucial compound having anti-inflammatory activity, anti-cancer activity and also showed benefit in diabetes [17, 18, 19].



Taraxerol (1)

Compound TC-2 was isolated from the fraction no 5 of $CHCl_3$ extract of the stem bark as yellowish oily liquid. It was as a purple color spot on the TLC (Silica gel F254) plate with vanillin-H_2SO_4 reagent followed by heating at 110°C for 5 minutes. The Rf value of TC-2 was 0.55 in dichloromethane-hexane (60:40) over Silica gel F_{254} plates. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of TC-2 showed a one proton multiplate at δ_H 3.65, was indicative of H-3 in a steroid nucleus. The absence of any olefinic proton signal between ∂_H 5.2-5.7 suggested that the double bond between C-5 and C-6 was absent, i.e., the compound was a dihydro analog of beta-sitosterol. However, a broad signal at δ_H 4.07 demonstrated the presence of another oxymethylene proton at C-6 while in case of) the oxymethylene at C-3 appeared at 3.40. Thus, the hydroxyl groups at H-3 and H-6 were assigned at beta position.

The ^{1}H spectrum of TC-2 exhibited two doublets (J=6.8 Hz) centered at δ_H 0.83 and 0.84 which could be attributed to the methyl groups of H3-26 and H3-27. The doublets (J=6.4 Hz) at ∂_H 0.89 was assigned to the methyl group H3-21. Two other methyl groups H3-18 and H3-19 were appeared as two singlets of three protons at δ_H 0.63 and 0.79 respectively. On this basis compound TC-2 was tentatively identified as stigmastane-3,6-diol (2) which was further confirmed by comparing with published data[20]. Stigmastane-3,6-diol compound can be used as a biological target for diabetes, inflammation and analgesia[21].



Stigmastane-3, 6-diol (2)

In the present study, the antimicrobial activities of different extracts from *T. catappa* were presented in tables below (Tables 1 and 2). The zones of inhibition produced by the petroleum ether, ethyl acetate and methanol soluble extracts of *T. catappa* were ranged from 7 mm to 13 mm. The methanol extract did not exhibit any significant activity against any bacterial strain used. Chandra et al found antibacterial activity from the leaf extract of *T. catappa*[18] whereas they also found better antibacterial activity against gram positive bacteria than bran negative bacteria[22].

Table 1. Antibacterial activities of the different fractions of *T. catappa* against gram positive bacteria

Gram positive bacteria	**Determination of zone of inhibition (mm) after 24 hours of incubation**			
	PEE (400 µg/disc)	EAE (400 µg/disc)	ME (400 µg/disc)	Ciprofloxacin (10 µg/disc)
Bacillus cereus	09	07	5	44
Sarcinana lutea	09	10	06	43
Staphylococcus aureus	11	--	5	43
Bacillus megaterium	7	--	4	44
Bacillus subtilis	12	9	6	44

[Note: Here "--" means no zone of inhibition]
PEE = Petroleum ether extract, EAE= Ethyl acetate extract, ME = Methanol extract

Table 2. Antibacterial activities of the different fractions of *T. catappa* against gram negative bacteria

Gram negative bacteria	**Determination of zone of inhibition (mm) after 24 hours of incubation**			
	PEE (400 µg/disc)	EAE (400 µg/disc)	ME (400 µg/disc)	Ciprofloxacin (10 µg/disc)
Vibrio mimicus	11	13	6	43
Salmonella typhi	9	8	4	43
Salmonella paratyphi	10	10	5	43
Shigella boydii	9	09	6	44
Shigella dysenteriae	12	11	6	44
Pseudomonas sp	09	10	6	44
Escherichia coli	12	11	6	44
Vibrio parahemolyticus	09	7	5	43
Kiebsiella sp.	10	09	4	44
Vibrio cholerae	09	08	5	44

The petroleum ether extract of *T. catappa* exhibited moderate sensitivity against gram positive bacteria like- *Bacillus subtilis, Sarcinana lutea, Staphylococcus aureus &* all gram negative bacteria. Weak activity was found against *Bacillus cereus* and *Bacillus megaterium.* The ethyl acetate extract exhibited moderate activity against gram positive bacteria - *Sarcinana lutae* and *Bacillus subtilis &* gram negative bacteria-*Vibrio mimicus, Kiebsiella sp., Pseudomonas sp, Salmonella paratyphi, Shigella dysenteriae, Shigella boydii and Escherichia coli. But Bacillus megaterium* and *Staphylococcus aureus* were found to insensitive. The methanolic extract showed moderate activity against gram positive bacteria like-*Sarcinana lutea, Staphylococcus aureus, Bacillus subtilis &* all gram negative bacteria. *Sarcinana lutea, Staphylococcus aureus, Bacillus subtilis, Vibrio mimicus, Salmonella typhi, Salmonella paratyphi, Vibrio cholera, Kiebsiella sp. showed* no significant sensitivity. Ciprofloxacin was used as a standard in these studies and all the extracts of *T. catappa* (bark) had significant *in vitro* antibacterial activity among the entire test samples.

The antifungal activity (Table 3) of different extract of *T. catappa* was also carried out by using the disc diffusion method with of incubation period of 48 hours at room temperature. In this test, griseofulvin was used as standard antifungal antibiotic that showed antifungal activity with a range of 10 mm to 12 mm. Different extracts demonstrated different antifungal activity by showing promising zone of inhibition against the fungi except *Saccharomyces cerevacae.*

Table 3. Antifungal activities of the different extracts of *T. catappa*

Fungi	Determination of zone of inhibition (mm) after 48 h incubation			
	PEE (400 µg/disc)	EAE (400 µg/disc)	ME (400 µg/disc)	Griseofulvin (25µg/disc)
Candida albicans	9	11	8	10
Aspergillus niger	8	9	7	12
Saccharomyces cerevacae	-	4	5	12

Table 4 represented the result of the brine shrimp lethality bioassay where vincristine sulphate was the positive control. The lethal concentration LC_{50} of the test was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration by means of regression analysis. However, varying degree of lethality to *T. catappa* was observed with exposure to different dose levels of the test samples. The degree of lethality was directly proportional to the concentration of the extracts ranging from moderate with the lowest concentration (0.781µg/ml) to significant with the highest concentration (400µg/ml). With the increase in concentration of test samples the mortality rate also increased gradually.

Table 4. Determination of LC_{50} of brine shrimp lethality bioassay for chloroform soluble crude extract of *T. catappa*

Sample No.	Conc. of plant crude extract (µg/ml)	Log C	% of mortality of brine shrimp			Mean	LC_{50}
			Expt. 1	Expt. 2	Expt. 3		
1	400.00	2.602	100	100	100	100	1.55 µg/ml
2	200.00	2.301	90	100	90	93.33	
3	100.00	2.000	90	90	80	86.66	
4	50.00	1.699	80	90	80	83.33	
5	25.00	1.398	80	70	80	76.66	
6	12.50	1.097	70	70	70	70.00	
7	6.25	0.796	70	60	70	66.66	
8	3.125	0.495	60	60	70	63.33	
9	1.56	0.194	60	60	60	60	
10	0.00	-----	00	00	00	00	

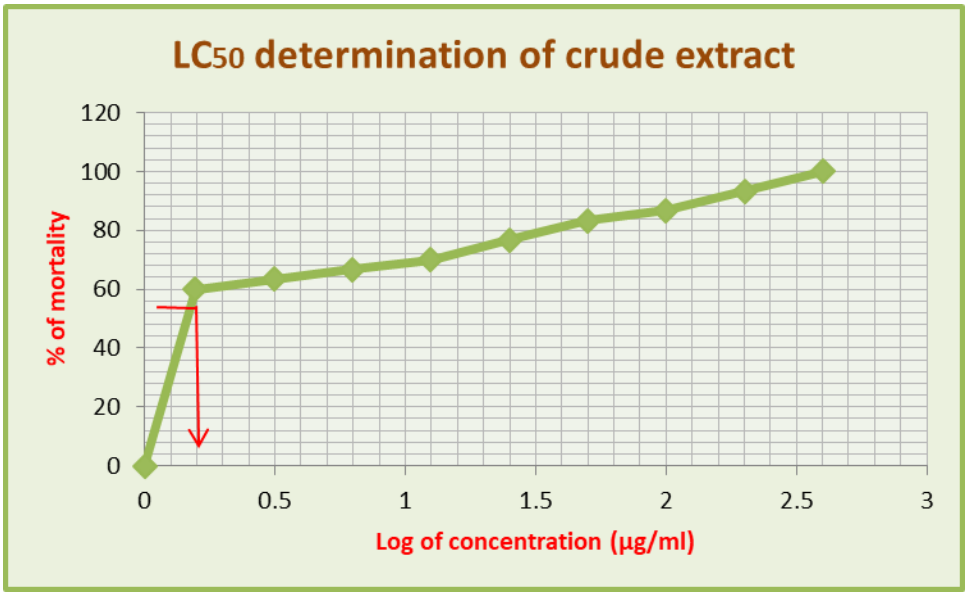


Figure 1. LC_{50} of brine shrimp lethality bioassay from chloroform soluble crude extract. Percent of mortality of brine shrimp directly proportional to the concentration of plant crude extract.

Table 5. Determination of LC_{50} of brine shrimp lethality bioassay for positive control vincristine sulfate

Sample No.	Conc. of vincristine sulfate (µg/ml)	Log C	% of mortality of brine shrimp			Mean	LC_{50}
			Expt. 1	Expt. 2	Expt. 3		
1	400.00	2.602	100	100	100	100	3.71 µg/ml
2	200.00	2.301	93	97	95	95.0	
3	100.00	2.000	88	90	92	90	
4	50.00	1.699	81	85	74	80	
5	25.00	1.398	74	70	66	70	
6	12.50	1.097	70	68	57	65	
7	6.25	0.796	62	60	37	53	
8	3.125	0.495	52	57	32	47	
9	1.56	0.194	46	47	27	40	
10	0.00	-----	00	00	00	00	

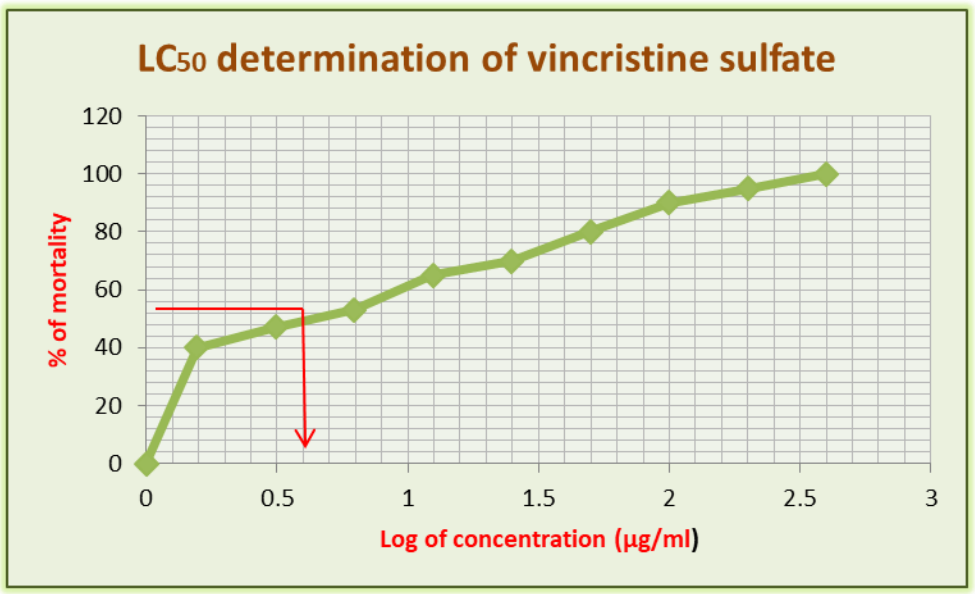


Figure 2. LC_{50} of Vincristine sulfate which used as a positive control (in cytotoxic activity) indicate that percent of mortality increased by increasing the concentration vincristine sulfate.

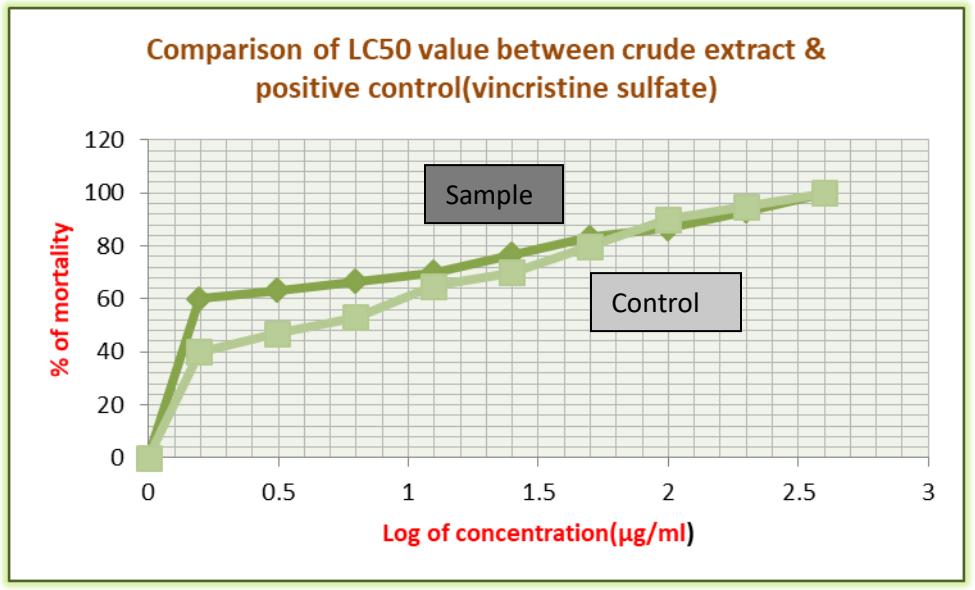


Figure 3. Comparison of LC_{50} value between chloroform soluble crude extract (bold green) & positive control (vincristine sulfate) (light green).

In this bioassay, the extract of *T. catappa* showed a significant cytotoxic activity (Table 5) in the brine shrimp lethality bioassay indicates that the compounds were biologically active. The percent of mortality of the brine shrimp nauplii was calculated for every concentration for chloroform soluble sample. A plot of log of concentration of the sample versus percent mortality was done. The LC_{50} values of chloroform soluble crude extract was found to be 1.55 µg/ml. Comparison with positive control vincristine sulfate signified that cytotoxicity exhibited by the test samples. From the results of this bioassay, it was predicted that the chloroform soluble crude extract possessed cytotoxic principles and have considerable cytotoxic potency.

Table 6. IC_{50} Value of Tert-butyl-1-hydroxytoluene

Serial No.	Absorbance of blank at 515 nm	Concentration (µg/ml)	Tert-butyl-1-hydroxytoluene at 515 nm.	% inhibition	IC_{50}(µg/ml)
1	0.346	500.00	0.034	90.17	
2	0.346	250.00	0.065	81.21	
3	0.346	125.00	0.078	77.46	
4	0.346	62.50	0.138	60.12	
5	0.346	31.25	0.163	52.89	17.88µg/ml
6	0.346	15.625	0.188	45.71	
7	0.346	7.813	0.207	40.17	
8	0.346	3.906	0.216	37.57	
9	0.346	1.953	0.238	31.21	
10	0.346	0.977	0.28	19.08	

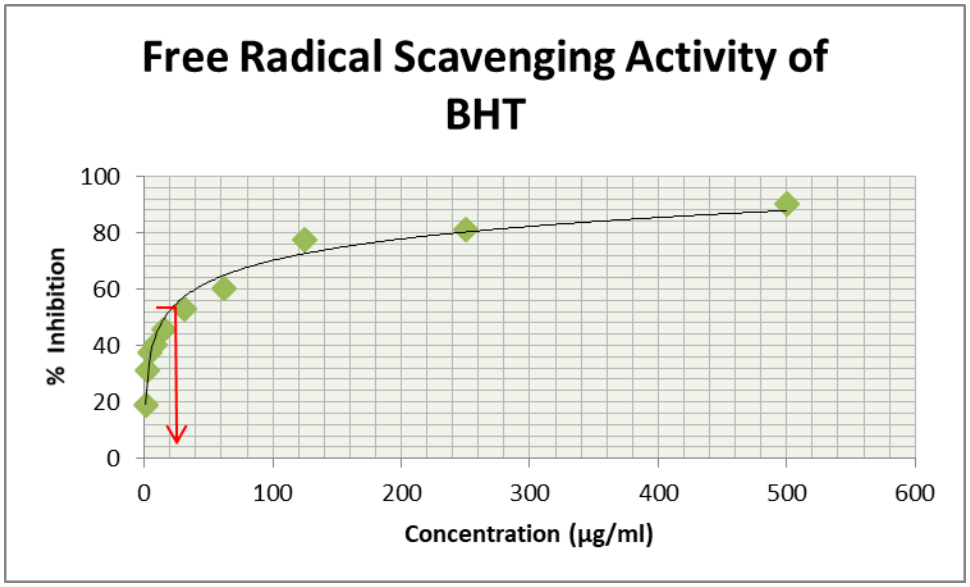


Figure 4. IC_{50} value of Tert-butyl-1-hydroxytoluene (BHT) was found 17.88µg/ml which used as a positive control for free radical scavenging activity

Table 7. IC_{50} Value of chloroform soluble crude extract of *T. catappa*

Serial No.	Absorbance of blank at 515 nm	Concentration (µg/ml)	Absorbance of Extract at 515 nm	% inhibition	IC_{50}(µg/ml)
1	0.346	500.00	0.105	69.65	
2	0.346	250.00	0.173	50.00	
3	0.346	125.00	0.213	38.44	
4	0.346	62.50	0.262	24.28	
5	0.346	31.25	0.302	12.72	255.0 µg/ml
6	0.346	15.625	0.320	7.51	
7	0.346	7.813	0.332	4.05	
8	0.346	3.906	0.340	1.73	
9	0.346	1.953	0.342	1.16	
10	0.346	0.977	0.345	0.065	

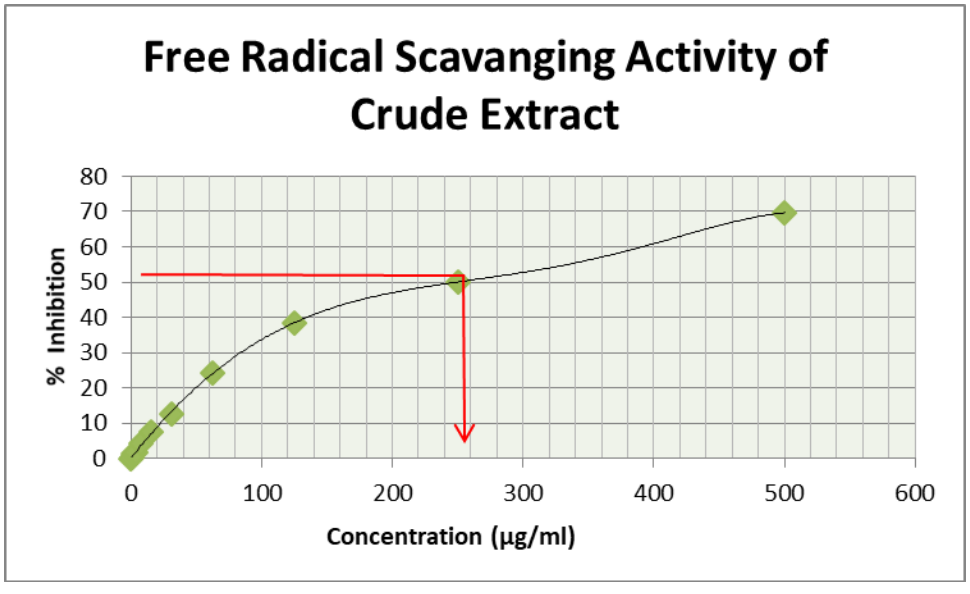


Figure 5. Free radical scavenging activity of crude extract from *T. catappa*, IC_{50} value of 255.0 µg/ml was found from chloroform soluble crude extract.

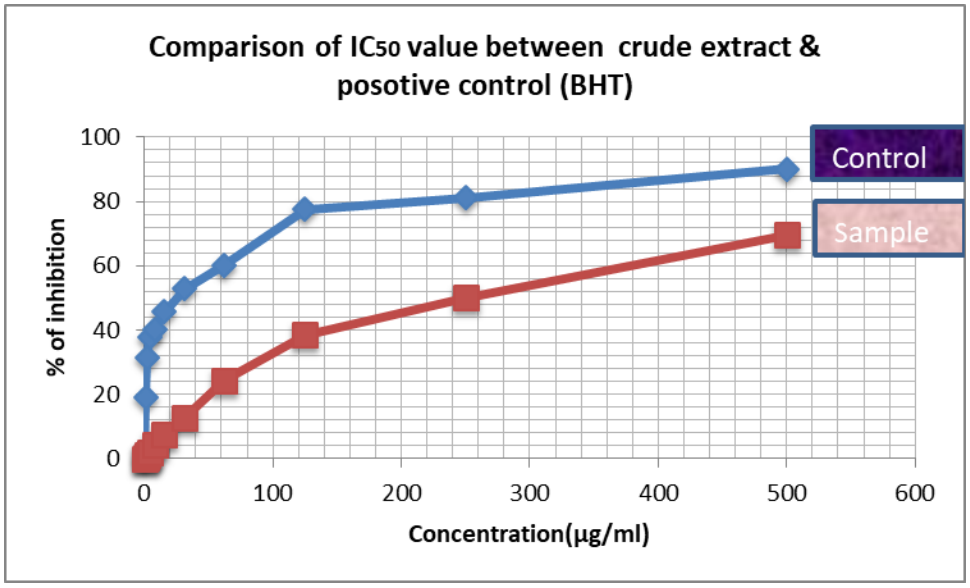


Figure 6. Comparison of IC_{50} value between chloroform soluble crude extract (Red) & positive control (BHT) (Blue).

Crude extracts of of *T. catappa* was subjected to free radical scavenging activity by the method of Auddy B et al[15]. Here BHT was used as reference standard, which IC_{50} value was found 17.88µg/ml. In this study, the crude extract demonstrated the highest free radical scavenging activity with IC_{50} value of 255.0 µg/ml (Table 6 and 7). So, the plant *T. catappa* exhibited a number of promising biological and pharmacological activities. Therefore, it can be a good source of natural medicine. So further research is necessary on this plant to achieve this goal.

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