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Some biochemical compounds of bryozoan, *Zoobotryon verticillatum* (Delle Chiaje, 1828) in the inner harbor of Visakhapatnam, east coast of India

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ABSTRACT

Zoobotryon verticillatum (Phylum: Ectoprocta) is a marine animal, which exist as colonies. It is widely distributed in inner harbor of Visakhapatnam. Z. verticillatum found attached to wood and ropes of sunken fishing vessels at fishing harbor as fouler. Preliminary screening of biochemical compounds in Z. verticillatum indicated that it is a rich source of alkaloids and steroids. The alkaloids detected were Quinoline, Purins and Quinazoline and their quantities (mg/500 mg sample) estimated were 73.090 mg, 155.235 mg and 271.675 mg respectively. The steroids detected were Norethindrone and Prednisone and their quantity (mg/25 mg sample) estimated was 12.0065 mg and 12.9935 mg respectively. These compounds are biologically active as cytotoxic, anti-malarial, neurotropic, androgenic, gonadotropin inhibiting and pregnancy inhibiting properties.

Key words: *Zoobotryon verticillatum*, HPLC, extraction, isolation, alkaloids, steroids, inner harbor of Visakhapatnam

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INTRODUCTION

Bryozoans (Ectoprocts) are aquatic invertebrates which are abundant in modern marine environments. These animals exist as colonies. The colony is always immovable attached to various substrates. There are about 22,000 fossil species and 5000 living species recorded in the world, of which 129 species of bryozoans recorded from the EEZ of India [18]. Rao, *et. al*., [27] reported forty-eight species belonging to 31 genera falling in to 24 families in the intertidal region of Visakhapatnam. Bryozoans produce remarkable varieties of chemical compounds, some of which may find use in medicines [26].

The majority of bryozoan metabolites isolated to date have been alkaloids [3]. *Z. verticillatum* has yielded 2,5,6, Tri Bromo N-Methylindole-3-Carboldehyde, which delays the metamorphosis in fertilized sea urchin eggs at low concentration [4,17]. There are several bioactive compounds isolated from various bryozoan species like 1 – vinyle – 8 - hydroxy – ß - carboline from *Catericella cribraria* [14]; Convolutamydine – A [31]; Convolutamydines B~D [11] from *Amathia convolute*; brominated alkaloids [Moris and Princep, 1999] [23]; Amathamide –A [22] from *Amathia wilsoni*; Pterocellins A and B from *Pterocella visiculosa* [2, 26]; bryostatins from *Bugula neritina* [29]; oxygenated sterols from *Crytosula pallasiana* [30]. These compounds showed cytotoxic, anti cancer and antimicrobial activity. The present study is an attempt to investigate the biochemical compounds from *Z. verticillatum* collected in the inner harbor of Visakhapatnam, Bay of Bengal, east coast of India.

MATERIALS AND METHODS

The present study was based on the bryozoan species, *Z. verticillatum* (one which forms big colonies) collected from wooden materials of sunken fishing vessels in the inner harbour of Visakhapatnam (Figure. 1) by help of local fishermen and carefully kept in a insulated box containing crushed ice at regular intervals (once in a month) from February 2011 to January 2012.

The collected samples were immediately brought to the laboratory and were washed properly with tap water after removing the ice and unwanted material (Ectocommensals etc). These samples were kept in deep freezer at -20^{o} C for further analysis. Fresh *Z. verticillatum* sample was used for preparation of solvent extract with petroleum spirit, chloroform, ethyl acetate, methanol and ethanol. The solvent extraction was used for preliminary screening of alkaloids, steroids and tannins by following the standard methods [5, 9, 30].

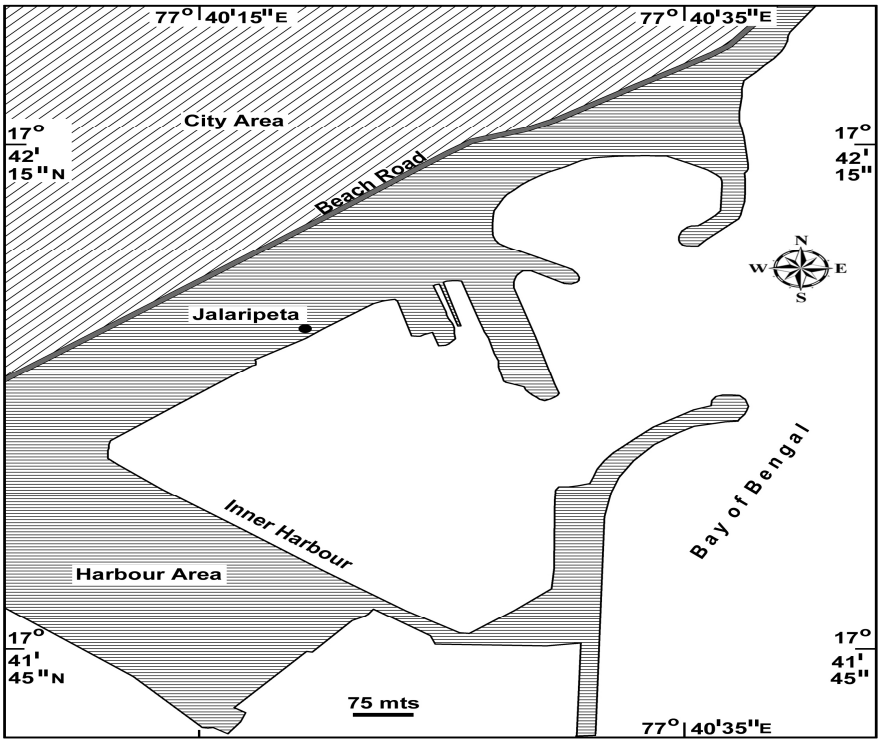


Figure 1. Map of the study area: Visakhapatnam Inner Harbour, Bay of Bengal

Solvent extraction

The animal tissue was kept in contact with solvent (petroleum sprit, chloroform, ethyl acetate, methanol and ethanol) at 1:3 ratio w/v in stoppered container for a defined 4-5 days period with frequent agitation until soluble matter is dissolved. The filtrate used for preliminary screening.

A. Methods for preliminary screening

i. Alkaloids

a) Dragendorff's test

Two drops of Dragendorff's reagent were added to the 2 ml of filtrate. Formation of orange or orange red colour precipitate indicated the presence of alkaloids.

b) Mayer's test

2-3 drops of Mayer's reagent was added to the few m1 of filtrate along the sides of the test tube. Formation of white or pale yellow precipitate indicated the presence of alkaloids.

c) Wagner's test

Two or three drops of Wagner's reagent were added to the few m1 of filtrate along the sides of the test tube. The formation of yellow or brown precipitate indicated the presence of Alkaloids.

ii. Steroids

a) Libermann-burchard test

2 m1 of filtrate was dissolved in acetic anhydride, heated to boiling, cooled and then 1 m1 of concentrated sulphuric acid was added along the sides of the test tube. Formation of green in colour indicated the presence of steroids.

b) Salkowski Test

2 m1 of filtrate was shaken with a few m1 of chloroform. To the chloroform layer, sulphuric acid was added slowly by the sides of test tube. Formation of yellow or reddish brown colour indicated the presence of steroids.

iii. Tannins

a) Dilute Ferric chloride test

Two drops of 5% ferric chloride solution was added to the 2 ml of filtrate. Green or blue colour indicated the presence of gallotannins, while brown colour indicated the presence of pseudotannins.

b) Lead acetate test

Three m1 of 10% lead acetate solution was added to the 2 ml of filtrate. Formation of bulky white precipitate indicates the presence of phenolic compounds.

B. Separation of compounds by HPLC-Technique

a) Extract preparation for alkaloid's separation

Extraction of sample by Cristiane, *et al.*, [6] procedure. The separation of alkaloids by HPLC conditions followed by James and Denise [12].

b) Extract preparation for Steroids separation

Extraction of sample was done by Gabi Schmidt and Hans Steinhart [8] method. The separation of steroids by HPLC conditions followed by James and William [13].

RESULTS

A.Preliminary screening

i.Alkaloids (Table 1)

a) Dragendorff's test

The alkaloids are present in petroleum spirit extract and ethyl acetate extract, but negative results obtained in methanol, ethanol and chloroform extracts.

b) Mayer's test

The alkaloids are present in methanol extract and ethanol extract, but negative results obtained in petroleum spirit, chloroform and ethyl acetate extracts.

c) Wagner's test

The test given positive results for alkolids from all five extracts.

ii.Steroids (Table 1)

a) Libermann-Burchard test

The steroids are present in methanol extract, ethanol extract, chloroform extract, ethyl acetate extract, but negative results obtained in petroleum spirit extract.

b) Salkowski test

The steroids are present in methanol extract, but negative results obtained in ethanol, chloroform, ethyl acetate and petroleum spirit extracts.

iii.Tannins (Table 1)

a) Ferric chloride test

The test given negative results for tannins from all five extracts.

b) Lead acetate test

The tannin's are present in ethanol extract, but negative results obtained in methanol, petroleum spirit, chloroform and ethyl acetate extracts.

B. Separation of compounds by HPLC

a. Alkaloids

The *Z. verticillatum* crude extract sample (500 mg.) was injected into HPLC using standard protocol. The following alkaloids identified were Quinoline, Purins and Quinazoline. The retention time for alkaloids was 2.606 min, 5.45 min and 8.54 min respectevly. The percentage of the Quinoline, Purins and Quinazoline in the extract of *Z. verticillatum* was 14.618 which correspond to 73.090 mg of the sample weight, 31.047 which correspond to 155.235 mg. of the sample weight and 54.335 which correspond to 271.675 mg. of the sample weight respectively (Table 2). The test sample chromatogram was correlated to the retention time of standard preparation of the chromatogram (Figures 2 & 3).

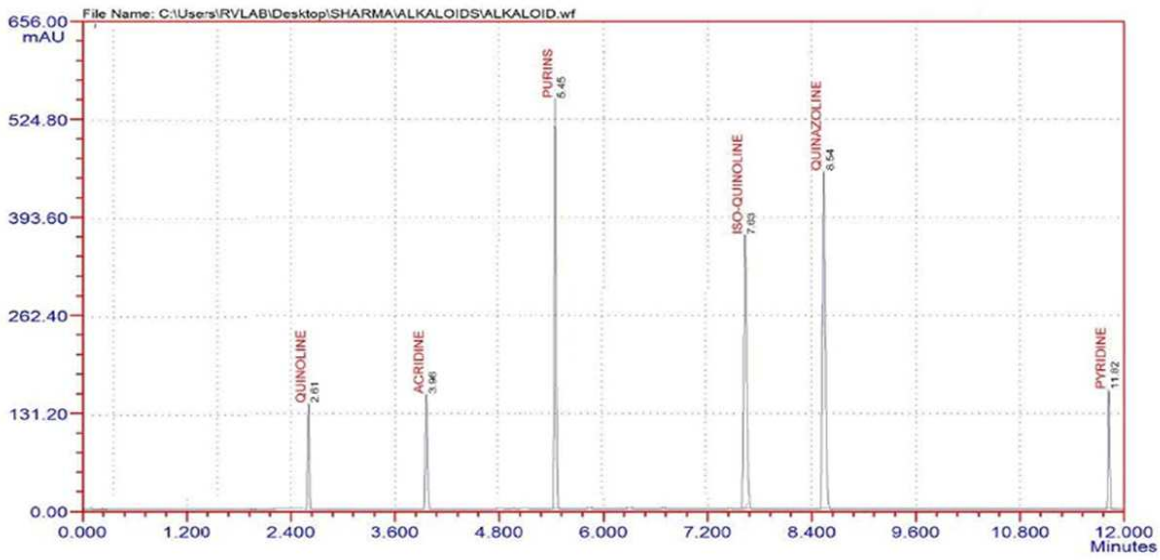
Table: 1. Results of biochemical compounds in *Z. verticillatum* for different tests

Name of the Test	Name of Extract				
	Methanol	Ethanol	Petroleum-Spirit	Chloroform	Ethyl Acetate
Alkaloids:					
a) Dragendorff's test	-	-	+	-	+
b) Mayer's test	+	+	-	-	-
c) Wagner's test	+	+	+	+	+
Steroids:					
a) Libermann-Burchard test	+	+	-	+	+
b) Salkowski test	+	-	-	-	-
Tannins:					
a) 5% Ferric chloride test	-	-	-	-	-
b) Lead acetate test	-	+	-	-	-

Note: (+): Present; (-): Absent.

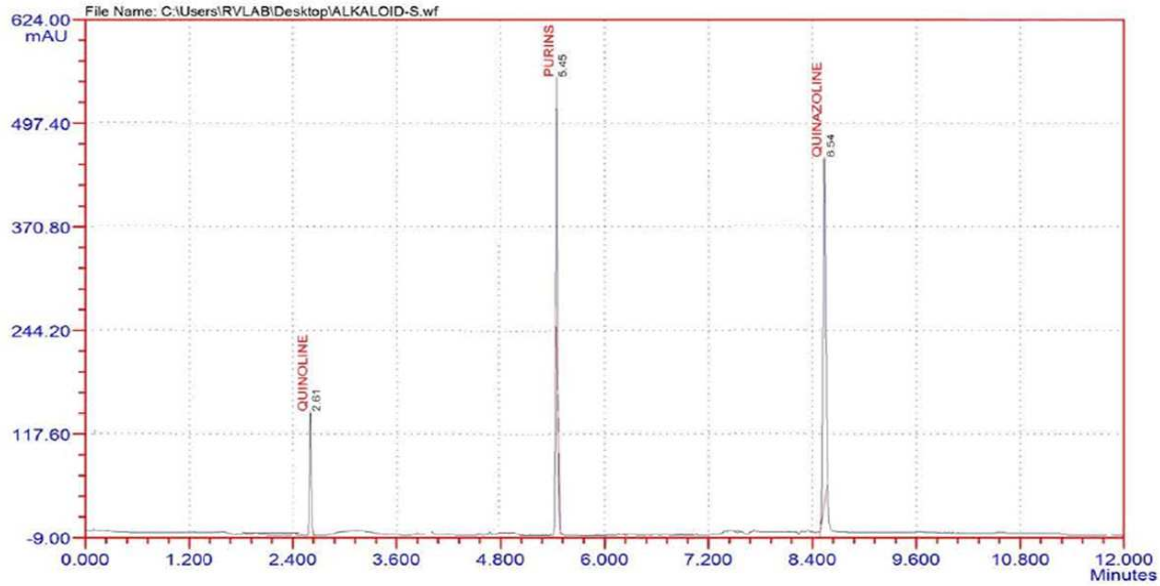
Table: 2. Qualitative and Quantitative separation of alkaloids by HPLC standard protocol

S. NO	Alkaloid	Retain- time	% of Alkaloid	Amount of Alkaloid
1	Quinoline	2.606	14.618	73.09mg
2	Purins	5.451	31.047	155.235mg
3	Quinazoline	8.541	54.335	271.675mg



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	QUINOLINE	2.60	15323	23277.9	6.478	0.91	58662
2	ACRIDINE	3.96	16535	30363.2	8.450	1.14	92830
3	PURINS	5.45	56270	89003.4	24.769	0.25	236737
4	ISO-QUINOLINE	7.63	36464	81930.6	22.800	1.18	230111
5	QUINAZOLINE	8.54	45848	107490.0	29.913	1.51	264521
6	PYRIDINE	11.82	17274	27275.1	7.590	0.70	1117736
	Sum:		187714	359340.2	100.0000		

Figure. 2: HPLC Report: - Chromatogram showing standard alkaloids

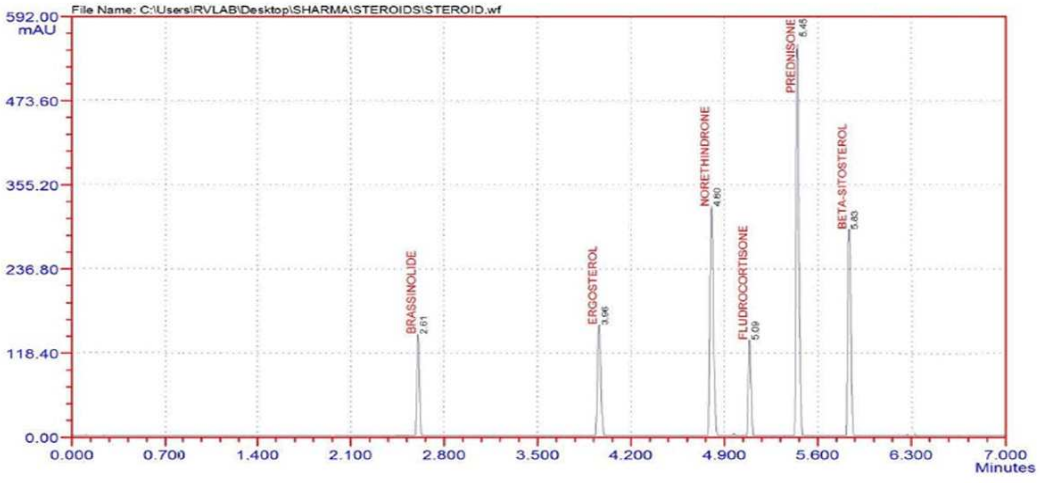


ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	QUINOLINE	2.606	15366	23476.4	14.618	0.87	57998
2	PURINS	5.451	34245	49860.5	31.047	1.00	279387
3	QUINAZOLINE	8.541	42468	87258.6	54.334	1.11	344399
	Sum:		92079	160595.5	100.0000		

Figure. 3: HPLC Report: - Chromatogram showing alkaloids in *Z. verticillatum*

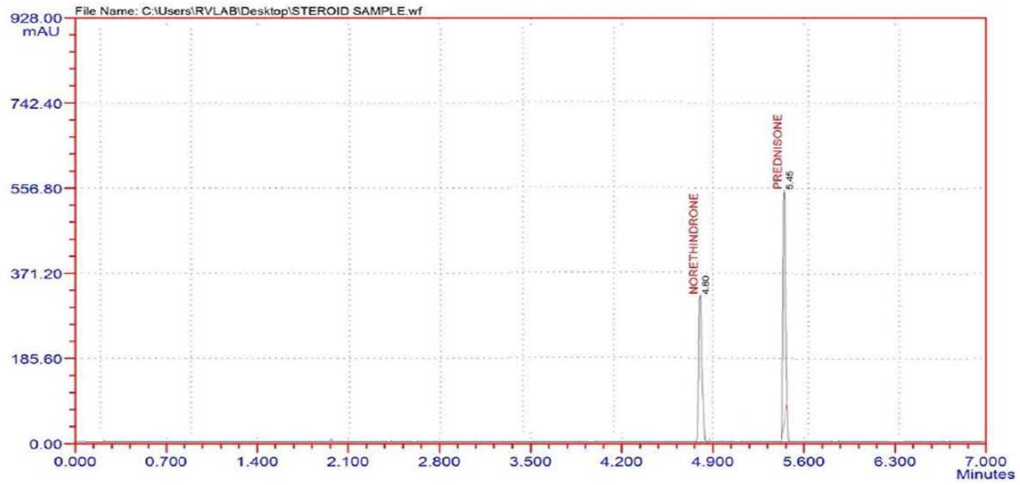
b.Steroids

The *Z. verticillatum* crude extract sample (25 mg.) was injected into HPLC using standard protocol. The steroids detected were Norethindrone and Prednisone. The test samples of steroids were eluted respectively at retention time of 4.805 min and 5.451 min. The percentage of the Norethindrone and Prednisone in the extract of *Z. verticillatum* was 48.026 which correspond to 12.0065 mg. of the sample weight and 51.974 which correspond to 12.9935 mg. of the sample weight respectively (Table 3). The test sample chromatogram was correlated to the retention time of standard preparation of the chromatogram (Figures 4 & 5).



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	BRASSINOLIDE	2.606	15315	23316.4	8.302	0.86	58407
2	ERGOSTEROL	3.963	16681	31933.2	11.370	1.52	85415
3	NORETHINDRONE	4.805	32979	61393.2	21.860	0.08	132767
4	FLUDROCORTISONE	5.091	14417	23193.8	8.258	0.90	199600
5	PREDNISONE	5.451	56177	86259.1	30.713	0.12	251207
6	BETA-SITOSTEROL	5.835	30193	54755.3	19.496	0.83	206306
	Sum:		165762	280851.0	100.0000		

Figure. 4: HPLC Report: - Chromatogram showing standard steroids



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	NORETHINDRONE	4.805	32881	59554.8	48.026	1.64	140252
2	PREDNISONE	5.451	50267	64450.5	51.974	1.02	360278
	Sum:		83148	124005.3	100.0000		

Figure. 5: HPLC Report: - Chromatogram showing steroids in *Z. verticillatum*

Table: 3. Qualitative and Quantitative separation of steroids by HPLC standard protocol

S.NO	Steroid	Retain- time	% of Steroid	Amount of Steroid
1	Norethindrone	4.805	48.026	12.0065mg
2	Prednisone	5.451	51.974	12.9935mg

DISCUSSION

The crude solvent extract of *Z. verticillatum* prepared by methanol, ethanol, petroleum ether, chloroform and ethyl acetate are shown alkaloids, steroids and tannins. Alkaloids: Quinoline, Purins and Quinazoline; Steroids: Norethindrone and Prednisone isolated from *Z. verticillatum* in the present study. These compounds and its derivatives were biologically active as cytotoxic, anti-malarial, neurotropic, androgenic, gonadotropin inhibiting and pregnancy inhibiting properties reported in earlier studies. Fournet *et. al*., [7] reported antiprotozoal activity of Quinoline alkaloids isolated from stem bark, root bark and leaves of *Galipea logiflora* showed an activity on promastigote forms of *Leishmonia Spp*. and epimastigote forms of *Trypanosoma cruzi* parasites. Zhongze Ma *et. al.*, [32] isolated novel Quinazoline, Quinoline alkaloids from the arial parts of *Peganum nigellastrum.* These compounds showed cytotoxic activity and DNA topoisomerase II inhibition. Michael [19, 20] reported an antibiotic producing isolate from a marine bacterium, *Altermonas sp.*, was found to contain two well known bacterial alkaloids, named as 2-Heptyl quinolin-4-ol and 2-pentyle quinolin-4-ol. The interesting observation in these studies was that the major metabolite was able to inhibit respiration in other bacteria, DNA and protein synthesis as well as bacterial motility at micro concentrations. It also inhibits growth of phytoplankton and diatoms.

According to Michael [19, 20], cytotoxicity-directed fractionation of an organic extract from the New Zealand ascidian *Pseudodistoma aureum* has led to the discovery of two interesting guanidine-containng Quanoline alkaloids possessing the furo, pyrano Quinoline skeleton. According to Michael [19, 20] dictyoquinazols A-C isolated from edible mushroom, *Dictyophora indusiata* were suggested to be possible therapeutics used in treating neurodegenerative diseases of brain. Kidder and Dewey [15] reported the biological activity of substituted purins and showed that the animal *Tetrahymena geleii*, requires an exogenous source of purin for growth. Manish *et. al.*, [16] studied synthesis, characterization and antimicrobial activity of some novel tri substituted purin bearing amino acid.

Powell and Elizabeth [25] studied the biological conversion of prednisone to prednisolone and plasma protein binding in liver disease. They suggested that the patients with acute hepatitis or active chronic liver disease, there is impairment of reduction of the II-OXO group of prednisone and also impaired ring-A reduction of prednisolone. Mohamed *et. al.*, [21] reported biotransformation of prednisone using human intestinal bacteria by anaerobic incubation and docking studies. According to Gordon, *et. al.*, [10] the effect of 7α-methylation on biological activities of Norethindrone is mainly Urotropic, Androgenic, Propegestatic, Gonadotropin inhibiting and pregnancy

inhibiting properties. Takada, *et. al.,* [28] reported pretreatment with low doses of norethindrone potentiates the osteogenic effects of fluoride on human osteosarcoma cells. Peter Alexandersen, *et. al.,* [23] observed that Norethindrone acetate enhances the antiatherogenic effect of17 β-Estradiol: a secondary prevention study of Aortic Atherosclerosis in ovaviectomized cholesterol-Fed Rabbits. Achintya Saha, *et. al.*, [1] studied the effect of oral contraceptive norethindrone on blood-lipid and lipid per oxidation parameters. The results reveal that Northindrone caused significant extent of lipid peroxidation. They suggested that ascorbic acid, a promising antioxidant, at equivalent human dose levels of 250 mg and 500 mg could significantly reduce Northindrone induced lipid peroxidation.

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