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Hepatoprotective Activity of Bixa orellana Linn Leaf Extracts

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Abstract

The present investigation was aimed at investigating the Hepatoprotective activity of Bixa orellana Linn. (Bixaceae) leaves. The dried leaf powder was subjected to successive Soxhlet extraction using petroleum ether, chloroform, ethyl acetate, methanol and water extracts were investigated for Hepatoprotective activity in Wistar rats using standard methods. In the present study, the animals were pretreated with test extracts before inducing liver damage with CCl_4. Seven days after acclimatization the rats were divided into seven groups (I-IX), each group consisting of six animals. All animals were kept on same diet for 7 days. The results showed that the Methanolic extract was found to be more potent as hepatoprotective than the reaming extracts of the Bixa orellana leaves.

Keywords: *Annatto, Soxhlet extraction, Arachis oil, Acute toxicity*

Introduction

The liver is the largest internal organ present in vertebrates and some other animals. It lies below the diaphragm in the thoracic region of the abdomen. It produces bile, an alkaline compound which aids in digestion, via the emulsification of lipids. It also performs and regulates a wide variety of high-volume biochemical reactions requiring very specialized tissues. The liver plays a central role in transforming and clearing chemicals and is susceptible to the toxicity from these agents. Certain medicinal agents when taken in overdoses and sometime even when introduced within therapeutic ranges may injure the organ. Other chemical agents such as those used in laboratories and industries, natural chemicals (e.g. microcystins) and herbal remedies can also induce hepatotoxicity. Chemicals that cause liver injury are called hepatotoxins.

Biochemical markers (i.e. alanine transferase, alkaline phosphatase and bilirubin) are often used to indicate liver damage. Liver injury is defined as rise in either (a) ALT level more than three times of upper limit of normal (ULN), (b) ALP level more than twice ULN, or (c) total bilirubin level more than twice ULN

when associated with increased ALT or ALP. Liver damage is further characterized into hepatocellular (predominantly initial Alanine transferase elevation) and cholestatic (initial alkaline phosphatase rise) types. However, they are not mutually exclusive and mixed type of injuries are often encountered [1-4].

Materials and Methods

Procurement of Chemicals

The solvent used were of Laboratory grade obtained from EMerck Ltd., Mumbai. Groundnut or Arachis oil was purchased from local market. All other chemicals of highest available purity were obtained from HiMedia Laboratories, Mumbai.

Collection of Plant Material

The leaves of *B. orellana* were collected from the wild growing tree in the Botanical Garden, Biotechnology Department in Kakatiya University, Warangal, India. Identification and confirmation was performed a qualified taxonomist. A specimen was deposited in the institutional herbarium. The collected plant material was made thoroughly free from any foreign organic matter. Leaves were separated, shade dried and powdered with laboratory mixer and sieved.

Preparation of Extract

The dried leaf powder was subjected to successive Soxhlet extraction using petroleum ether (60-80°C), chloroform, ethyl acetate, methanol and water each for 6 h. The obtained solvent extracts were concentrated in vacuo using rotary vacuum evaporator and dried in desiccators [5].

Animals

The healthy Wistar rats of either sex, approximately the same age and weighing about 150-180 g used for the study were obtained from Mahaveer Enterprises, Hyderabad. They were fed with standard chow diet and water *ad libitum*. The animals were housed in polypropylene cages maintained under standard environmental conditions (12 h light/12 h dark cycle; 25 ± 3ºC, 35-60% relative humidity). The animals were treated strictly according to the CPCSEA guidelines and the study was conducted after obtaining permission from Institutional Animal Ethics Committee (IAEC).

Acute Toxicity and Gross Behavioral Study

The rats were fasted overnight, divided into groups (n=6) and were orally fed with increasing doses (250, 500, 750 and 1000 mg/kg body weight) of petroleum ether, methanol and aqueous extracts suspended in Groundnut (Arachis) oil. After administration of the extracts, the animals were observed during first 2 h for their gross behavioral changes and once in 30 min for next 4 h and then once in 24 h for next 72 h to find out percentage mortality [6-8].

In the present study, the animals were pre-treated with test extracts before inducing liver damage with CCl_4. Seven days after acclimatization the rats were divided into seven groups (I-VII), each group consisting of six animals. All animals were kept on same diet for 7 days.

Biochemical Parameters

The following biochemical parameters were evaluated in the serum [6-8].

a. Total serum bilirubin (TBL)
b. Alkaline Amino Transferase (ALT)
c. Aspartate Amino Transferase (AST)
d. Serum Alkaline Phosphatase (ALP)
e. Total Protein Levels (TPL)
f. Albumin Levels (ALB)

A. Estimation of Total Bilirubin

Method: It is carried out by Jendrassik and Grof's method.

Principle: Bilirubin reacts with diazotized sulfanilic acid in acidic medium to form azobilirubin concentration, a pink colored complex whose absorbance is proportional to bilirubin concentration. The estimation of total

bilirubin is carried out in the presence of an activator.

Preparation of Test Solution

To a clean test tubes, 1.0 ml of Diazo A (1) and 0.1 ml of Diazo B (2) reagents, 1.0 ml of activator (3), 2.5 ml of distilled water and 0.2 ml of serum were added. Correspondingly, a control was run in the similar manner without addition of Diazo B. The tubes were mixed well and kept in dark for 5 min. The optical density (absorbance-A) of tests (T1-T2) and then corresponding controls were read at 540 nm using spectrophotometer against distilled water. Similarly, the optical density of the artificial standard (A of standard) was read against distilled water. Serum bilirubin levels were calculated based on the following formula and expressed in mg per ml.

$$\text{Total serum Bilirubin in mg\%} = \frac{\text{A of T1-T2}}{\text{A of standard}} \times 10 \text{ n (std. conc)}$$

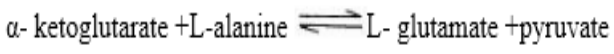
A of T1=Absorbance of test 1

A of T2=Absorbance of test 2

B. Estimation of Alanine Transaminase (ALT) Levels

Method: The ALT activity was carried out by the Reitman and Frankel method.

Principal: Alanine Transaminase catalyses the following reaction:



Pyruvate so formed is coupled with 2,4-Dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydrazone, which gives brown color in alkaline medium and this can be measured calorimetrically.

Preparation of Standard Solution

Five clean and dry test tubes were taken and marked as 1,2,3,4 and 5. Added 0.5 ml, 0.45 ml, 4 ml, 0.35 ml and 0.3 ml of Reagent 1 (Buffered Alanine α-KG substrate) to the test tubes respectively. then added 0.05 ml, 0.1 ml, 0.15 ml, 0.2 ml of Reagent 4 (working pyruvate standard) respectively. Finally, added to each test tube, 0.1 ml distilled water and 0.5 ml of Reagent 2 (2,4-DNPH color reagent). Then mixed well and allowed to stand at room temperature (15-30^{o}C) for 10 minutes and the O.D of all the five tubes were measured against purified water on spectrophotometer at 505 nm. ALT levels were obtained by extrapolation of the standard curve.

Preparation of Test Solution

Clean dry test tubes were taken and suitably marked. Then 0.25 ml of Reagent 1 (Buffered alanine α-KG substrate) was added to each test tube and incubated at 37^{o}C for 5 min. To this, 0.05 ml of serum was added, mixed and incubated at 37^{o}C for 30 min. Then 0.25 ml of Reagent (2) (2,4-DNPH) (dinitrophenyl hydrazine) color reagent was added, mixed well and allowed to stand at room temperature for 20 min. To this 2.5 ml of 0.4N NaOH solution-I was added. Mixed well and allowed to stand at room temperature for further 10 min for complete development of brown color. Optical density of the solution was read in spectrophotometer at 505 nm against blank (purified water). ALT levels were obtained by extrapolation of the standard curve.

C. Estimation of Aspartate Transaminase (AST) Levels

Method: it was carried out by Reitman and Frankel method.

Principle: Aspartate transaminase catalyses the following reaction:

Preparation of Standard Solution

Clean dry test tubes were taken and marked as 1, 2, 3, 4, and 5. then 0.5 ml, 0.45 ml, 0.4 ml, 0.35 ml, and 0.3 ml of Reagent 1 (Buffered Alanine α-KG substrate) were added to each test tube respectively. then added 0.05 ml, 0.1 ml, 0.15 ml, 0.2 ml, of Reagent (4) (working pyruvate standard) to test tubes labeled as 2 to 5. Finally, 0.1 ml of distilled water and 0.5 ml

of Reagent (2) (2,4-DNPH color reagent) were added to each test tube. Mixed well and allowed to stand at room temperature (15-30°C) for 10 min and the O.D of all the five tubes were measured against purified water on spectrophotometer at 505 nm.

Preparation of Test Solution

Clean and dry test tubes were taken and marked suitably, 0.25 ml of Reagent (1) (Buffered Alanine α-KG substrate) was added to each test tube and incubated at 37°C for 5 min. To this, 0.05 ml of serum was added, mixed well and incubated at 37°C for 30 minutes. then 0.25 ml of Reagent (2) (2,4-DNPH) (dinitrophenyl hydrazine) color reagent was added, mixed well and allowed to stand at room temperature for 20 min. To this 2.5 ml of 0.4N NaOH solution-I was added. Mixed well and allowed to stand at room temperature for further 10 min for complete devolvement of brown color. Optical density of the solution was read in spectrophotometer at 505 nm against blank (purified water). AST levels were obtained by extrapolation of the standard curve.

D. Estimation of Serum Alkaline Phosphatase Levels

Method: it was carried out by modified King and King's method.

Principal: Serum ALP hydrolyzes at pH 10.0. The phenol so formed reacts with 4-aminopyrine in alkaline medium in presence of oxidizing agent potassium ferricyanide to form a red colored complex whose absorbance is proportional to the enzyme activity.

Preparation of Blank Solution

To a clean and dry test tube labeled as blank, 1.0 ml of buffered substrate and 3.1 ml of deionized water was added. The test tube was incubated for 3 minutes at 37°C and added 2.0 ml of color Reagent (2). The absorbance A (B) was measured at 510 nm by using spectrophotometer.

Preparation of standard solution:

1.0 ml of buffered substrate and 3.1 ml of deionized water was added to a clean dry test tube and incubated for 3 min at 37°C. To this 0.1 ml of phenol standard was added. Then it was incubated for 15 minutes at 37°C. Finally, 2.0 ml of color Reagent (2). The absorbance A(S) was measured at 510 nm by using spectrophotometer.

Preparation of Control Solution

1.0 ml of buffered substrate and 3.1 ml of deionized water was added to a clean dry test tube and incubated for 3 min at 37°C. Finally, 2.0 ml of color Reagent (2). The absorbance A(S) was measured at 510 nm by using spectrophotometer.

Preparation of Test solution

1.0 ml each of working buffered substrate (phenyl phosphate) and distilled water were taken in clean dry labelled test tubes. Mixed well and incubated for 3 min at 37°C. To this, 0.1 ml of serum (test) was added, mixed well and incubated for 15 minutes at 37°C. Then 2.0 ml of color reagent was added and thoroughly mixed. The absorbance was measured at 510 nm by A (T) using spectrophotometer ALP levels were calculated based on the following formula and expressed in KA units/dl.

$$\text{Serum ALP in KA Units/dl} = \frac{A(T) - A(C)}{A(S) - A(B)} \times 10$$

KA = Kind and Anderson units/dl

A (T)=Absorbance of Test

A (C)=Absorbance of Control

A (S)=Absorbance of Standard

A (B)=Absorbance of Blank

E. Estimation of Total Protein Levels

Method: it was carried out by the Biuret and BCG dye binding method.

Principle: protein binds with copper ions in the alkaline medium of Biuret reagent and produces a purple colored complex. Whose absorbance is proportional to protein concentration.

Preparation of Blank Solution

Pippeted out 1 ml of Biuret reagent and 2 ml of distill water into a clean-labeled test tube. Mixed well and incubated at 37°C for 10 min. Then measure the absorbance A(S) at 555 nm using a spectrophotometer.

Preparation of Standard Solution

1 ml of Biuret reagent and 2 ml of distilled water and 0.05 ml of the standard protein provided with the kit were taken in a clean dry labeled test tube. Mixed well and incubated at 37°C for 10 min. Then measure the absorbance A(S) at 555 nm using a spectrophotometer.

Preparation of Test Solution

1 ml of Biuret reagent and 2 ml of distilled water followed by 0.05 ml of test solution were taken in a clean dry labeled test tube. Mixed well and incubated at 37°C for 10 min. Then measure the absorbance A(T) at 555 nm using a spectrophotometer.

The Total protein was calculated based on the following formula:

$$\text{Total protein in g\%} = \frac{\text{A of (T)}}{\text{A of (S)}} \text{X Std. Conc.}$$

A (T)=Absorbance of Test

A (S)=Absorbance of Standard.

Std. Conc.=7.1 (given in kit)

F. Estimation of Albumin Levels

Method: it was carried out by the Biuret and BCG dye Bining method.

Principle: Albumin in a buffered medium bind with bromocresol green (BCG) and produce a green color whose absorbance is proportional to the albumin concentration.

Preparation of Blank Solution

1 ml of buffered dye reagent and 2 ml of distilled water were taken in a clean-labelled test tube. Mixed well and immediately measured the absorbance A(B) at 630 nm.

Preparation of Standard Solution

1 ml of buffered dye reagent and 2 ml of distilled water and followed by 0.01 ml of standard (3) reagent protein provided with the kit were taken in the test tube. Mixed well and immediately measured the absorbance A(S) at 630 nm.

Preparation of Test Solution

1 ml of buffered dye reagent (2) and 2 ml of distilled water and followed by 0.01 ml of test sample (serum) was taken in clean labeled test tube. Mixed well and the absorbance A (T) was measured immediately on spectrophotometer at 630 nm.

Albumin activity was calculated based on the following formula:

$$\text{Albumin in g\%} = \frac{\text{A of (T)}}{\text{A of (S)}} \text{ X Std. Conc.}$$

A (T)=Absorbance of Test

A(S)=Absorbance of Standard.

Std. Conc.=5.4% (given in kit)

Histopathological Studies

The liver is made up of hepatocytes and specialized cells called kupffer cells interspersed with sinusoids. it is supplied with branches of bile duct. The normal hepatocytes have intact plasma membrane. While in the event of viral infection /disease state or when drug or chemicals affect liver cells there will be changes in the permeability of plasma membrane. disruption of cells is caused by excessive formation of fibrotic tissue eventually leading to necrosis.

Histopathological studies could be carried out to assess the degree of damage. This is done by staining the fine section of liver isolates and recording the degree of fibrosis (scoring from 0-4).

After the animals are sacrificed, livers were taken out and washed with normal saline (0.9%). Then, 2-3 pieces of approximately 6 cu.mm size were cut and fixed in phosphate buffered 10% formalin solution. After embedding in paraffin wax, thin sections of 5 µm thickness of liver tissue were cut and stained with haemotoxylin-eosin stain [9-10].

Processing of Liver Tissue

Liver tissues were taken out from fixing solution and dehydrated for 30 min each in 30, 50, 70, 90, & 100% alcohol successively. to remove the alcohol from the dehydrated tissues, they were kept for 30 minutes each in alcohol l: xylene (1:1) followed by pure xylene. The tissue was then kept in xylene: paraffin wax mixture (1:1) for 1 hour and then in molten paraffin wax at 62^{o}C., after which they were trimmed and mounted on wooden blocks for thin sectioning. Hand microtome (Yorco precision rotary microtome, model no YS1114) was used to cut thin sections of liver tissues of 5 µm thickness.

Staining and Mounting of Liver Tissues

Ribbons of thin sections of liver tissues were placed in rows on clean glass slides previously coated with albumin-glycerine mixture and few drops of water added to let the sections float. The slides were heated on hot plate to fix liver sections onto the slides. The slides were then placed for 5 min each in xylene to remove wax, then in absolute alcohol to remove xylene from the liver sections. Hydration of liver sections was attained by keeping them in descending series of alcohol and water mixtures (90%, 70%, 50%, 30% alcohol and in pure water) for three minutes each. Hydrated sections were stained with haemotoxylin stain for one minute and washed in running tap water to remove excess stain. Liver sections were dehydrated again by keeping in ascending of alcohol–water mixtures (30%, 50%, 70%, and 90% alcohol) for 1 min. After that, the sections were kept for 5 min each in absolute alcohol and then in xylene. Finally, the stained liver sections were mounted in DPX and viewed under optical microscope for histological examination (**Table 1**).

All the rats were anaesthetized with thiopentone sodium (60 mg/kg i.p.) 36 h after administration of CCl_4. Blood was collected from common carotid artery by carefully opening the neck region of the rat. After blood collection, the blood samples were allowed to coagulate at room temperature for at least one hour. Serum was separated by centrifugation at 3000 rpm for 30 minutes and then analysed for total bilirubin, direct bilirubin, ALT, AST, ALP, total protein and albumin levels. The animals were then dissected and the livers were carefully removed and washed with 0.9% saline solution and preserved in formalin solution (10% formaldehyde) for histopathological studies [11-12].

Results and Discussion

In acute toxicity study, all the animals were found to be surviving after 72 h. This indicates that the extracts were found to be safe up to the dose levels studied. Since, all the animals survived at a dose of 1000 mg/kg body weight, the LD_{50} of the extracts will be >1000 mg/kg body weight. No major behavioral changes were observed during this period of study. The animals showed mild sedative effect upon administration of all the extracts (**Table 2**).

From **Table 3**, **Figure 1** and **Figure 2**, it was observed that methanolic extract (500 mg/kg) showed significant hepatoprotective activity comparable to that of standard (100 mg/kg). Hence it can be said that methanolic of *Bixa orellana* have potent hepatoprotective activity when compared with that of petroleum ether and aqueous extracts.

Conclusion

From the results, it can be concluded Methanolic extract found to be more potent as hepatoprotective that the *Bixa orellana* leaves. However, the activity was not comparable in terms of quantitative activity elicited by standard drug. This could be due to the use of crude extracts. Hence, isolation of active principles will be advantageous to produce novel bioactive constituents from these extracts which may possess more significant activity.

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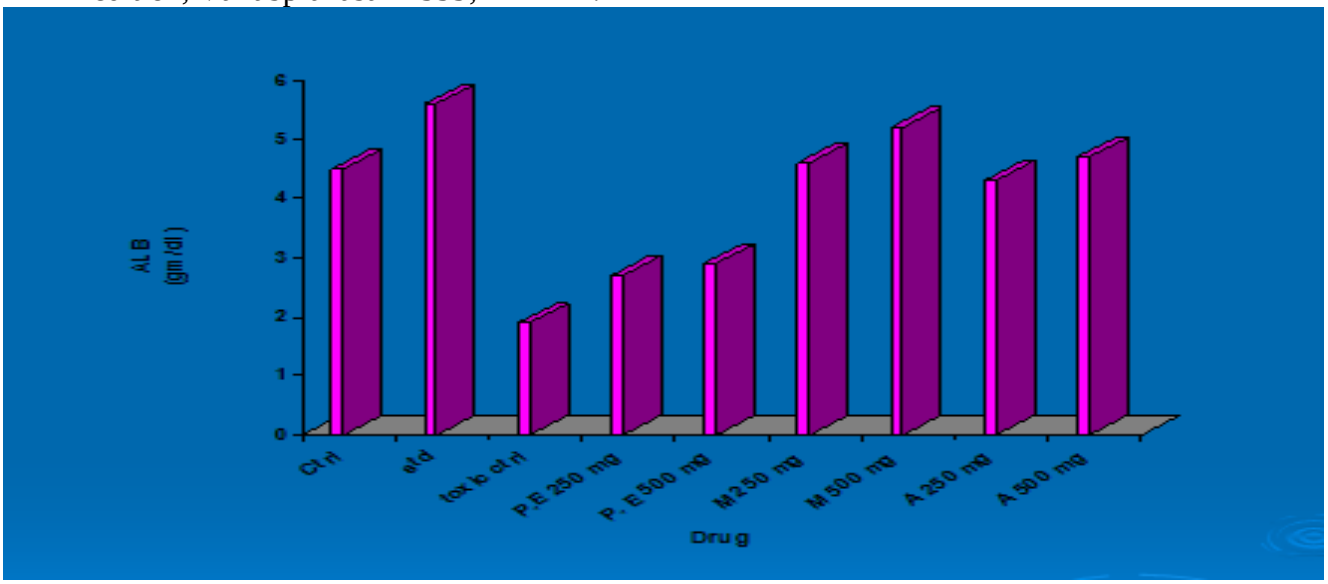
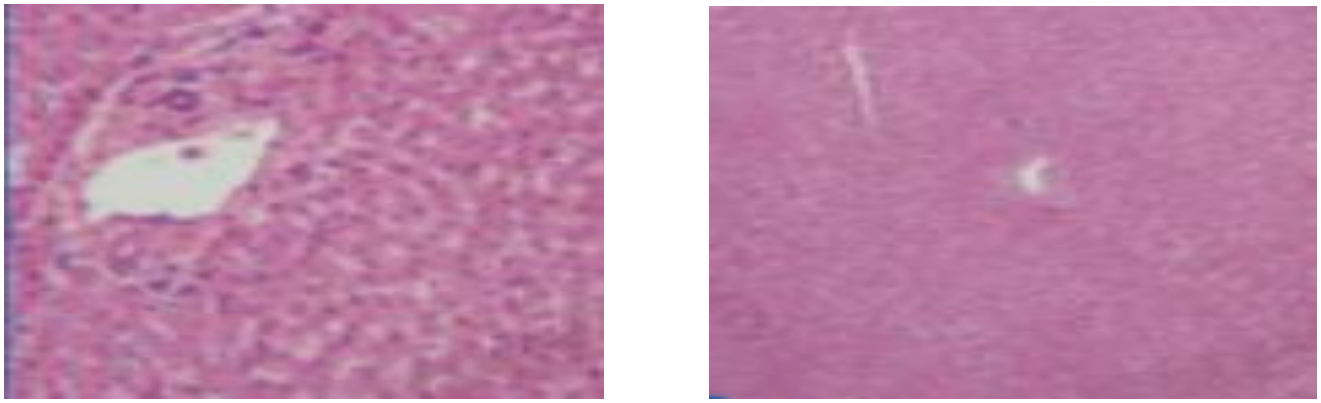


Figure 1: Effect of leaf extracts of Bixa orellana on ALB levels on CCl_4 induced liver toxicity

(A) **(B)** **(C)**



(D) **(E)**

Figure 2: Histopathological studies (A) Control (B) Standard (C) Toxic control (D) Methanolic extract 250 mg/kg (E) Methanolic extract 500 mg/kg

Table 1: Division of animals for hepatoprotective activity of *Bixa orellana*

Group	Extract
Group I (Control)	Arachis oil
Group II (Toxic control)	5 ml/kg of 50% v/v CCl_4 in olive oil I.P. on the seventh day.
Group III (Standard)	100 mg/kg of silymarin P.O. for seven days, followed by CCl_4 administration I.P. on the seventh day.
Group IV	Petroleum ether extract (250 mg/kg)
Group V	Petroleum ether extract (500 mg/kg)
Group VI	Methanolic extract (250 mg/kg)
Group VII	Methanolic extract (500 mg/kg)
Group VIII	Aqueous extract (250 mg/kg)
Group IX	Aqueous extract (500 mg/kg)

Table 2: Acute toxicity studies

Extracts	Group	Dose (mg/kg)	No of mice each group	After 4 hours	After 24 hours
Petroleum ether	I	250	6	6	6
	II	500	6	6	6
	III	750	6	6	6
	IV	1000	6	6	6
Methanol	I	250	6	6	6
	II	500	6	6	6
	III	750	6	6	6
	IV	1000	6	6	6
Aqueous	I	250	6	6	6
	II	500	6	6	6
	III	750	6	6	6
	IV	1000	6	6	6

Table 3: Hepatoprotective activity; effect of the parameters

Groups	Dose (mg/kg)	ALT (U/ml)	AST (U/ml)	ALP (KA units/ml)	TBL (mg/dl)	TPL (mg/dl)	ALB (gm/dl)
Control	-	70 ± 0.05	49±0.05	62 ± 0.05	0.65 ± 0.04	6.8 ± 0.06	4.5 ± 0.06
Standard (silymarin)	100	84 ± 0.03	58 ± 0.06	69 ± 0.04	0.75 ± 0.08	7.9 ± 0.04	5.6 ± 0.01
CCl_4	-	105 ± 0.07	92 ± 0.04	89 ± 0.08	0.45 ± 0.07	3.1 ± 0.06	1.9 ± 0.03
Petroleum ether	250	98 ± 0.04	74 ± 0.05	76 ± 0.07	0.49 ± 0.04	4.3 ± 0.05	2.7 ± 0.05
Petroleum ether	500	95 ± 0.05	70 ± 0.07	74 ± 0.04	0.52 ± 0.05	4.5 ± 0.03	2.9 ± 0.06
Methanol	250	79 ± 0.02	55 ± 0.06	63 ± 0.05	0.64 ± 0.04	6.8 ± 0.06	5.2 ± 0.05
Methanol	500	75 ± 0.03	52 ± 0.02	59 ± 0.04	0.70 ± 0.01	7.8 ± 0.05	4.6 ± 0.02
Aqueous	250	82 ± 0.03	58 ± 0.08	66 ± 0.01	0.59 ± 0.02	5.5 ± 0.04	4.3 ± 0.09
Aqueous	500	80 ± 0.08	61 ± 0.06	70 ± 0.02	0.62 ± 0.07	5.8 ± 0.06	4.7 ± 0.07

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