

Evaluation of Antibacterial Activities and Extraction of Phytochemical Components from Leaf of *Withania somnifera* (Gizawa)

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Abstract

*Nowadays, the uses of medicinal plants are more diverse to prevent human health care, in which medicinal plants are powerful inhibition of microorganisms cause disease. The study is conducted to assess evaluation of antibacterial activity, phytochemical screening and methods of extracting secondary metabolites of a traditionally used medicinal plant, Withania somnifera. The plant has been extracted using 80% aqueous ethanol by maceration technique, and 16.50 g of brown ethanol crude were obtained. The crude ethanol suspended in water and partitioned within different polarity of solvents (n-hexane, ethylene acetate, and n-buthanol), respectively. During partitioning, 7.5 g of ethylene acetate were collected after concentrated using a rotary evaporator and few grams of n-hexane were obtained. The study revealed phytochemical components like alkaloids, flavonoids, phenols, tannins, glycosides, terpenoids and steroids. The antibacterial properties of the crude extracts from Withania somnifera were tested against two gram-negative (*E. coli* and *K. pneumonia*) and two gram-positive (*S. aureus* and *S. pyogenes*), in which the active tie sample was administered at concentrations of 50 mg/ml, 75 mg/ml, and 100 mg/ml in total. During antibacterial tests, all the extracts exhibited strong antibacterial properties at high concentrations but, various at low concentration.*

Introduction

Plants generate numerous organic compounds, commonly referred to as metabolites, due to their metabolic activities. These metabolites are categorized into primary and secondary metabolites. Secondary metabolites are distinct from primary metabolites due to their limited presence within the plant kingdom. Medicinal plants are increasingly emphasized due to their potential to provide numerous undeniable advantages to individuals, particularly in the fields of medicine and pharmacological research. The therapeutic properties of these plants stem from phytochemical components that induce specific pharmacological effects on pathogen growth¹. Phytochemicals are naturally occurring compounds generated by organisms, primarily plants, including alkaloids, glycosides, flavonoids, tannins, steroids, terpenoids, and phenols, which are secondary metabolites that act as protective agents against microorganisms². Globally, including in Ethiopia, traditional medicinal plants have been widely used since ancient times to treat diseases and infections. Traditional medicine in Ethiopia relies on native knowledge and has been used for a long time to treat different illnesses³. For centuries, individuals have depended significantly on conventional medicine to address numerous physical and mental disorders. Around 80% of individuals in the nation continue to depend on traditional medicine as their primary healthcare system³, and according to WHO, traditional medicinal plants is the total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures and nations, as well as popular in the developing countries and its rapidly, spreading in the developed nations⁴.

In Ethiopia, a variety of plant species serve as medicinal plants and *Withania somnifera* is widely utilized among nearly the entire population of Ethiopia. *Withania somnifera*, referred to as Gizewa in Amharic

and Hunzo in Afan Oromo in Ethiopia, is part of the *Solanaceae* family⁵. *W. somnifera* is a tough grey, somewhat hairy evergreen bush that reaches approximately 1.5 m in height and possesses relatively long tuberous roots⁶. The small, yellowish-green flowers may be seen singly or clustered together. The fruit is rounded, smooth, and soft, containing numerous seeds; it turns orange-red upon ripening and is surrounded by a membranous shell^{7,5}. *W. somnifera* occurs in the arid sections of lowland regions of Arsi, North Shewa, Gojam, Wallo, Bale, and Tigray in Ethiopia.

Historically, the plant has served as an antioxidant, adaptogen, aphrodisiac, astringent liver tonic, anti-inflammatory substance, and more recently for managing ulcers, bacterial infections, venom toxins, and age-related dementia⁸. *W. somnifera* has served as a significant herb in Ayurvedic and traditional medicine for more than 3000 years. The plant roots are believed to enhance health and longevity by improving disease resistance, slowing the aging process, rejuvenating the body in weakened states, boosting an individual's ability to withstand negative environmental influences, and fostering a sense of mental engagement⁹. Clinical trials and animal studies endorse the application of *W. somnifera* for addressing anxiety, cognitive and neurological conditions, hyperlipidemia, inflammation, and Parkinson's disease¹⁰. The main objective of this study was to investigate in vitro isolation of secondary metabolites based on different polarity of solvent of secondary metabolites and evaluation of its antibacterial activity on two gram-negative (*E. coli* and *K. pneumonia*) and two gram-positive (*S. aureus* and *S. pyogenes*) bacteria.

Material and methodology

Plant collection

In December 2024, the mature and health *W. somnifera* plant species were collected from a family garden in Sinana woreda. The Sinana woreda is situated in the Bale zone of the Oromia region, southeast of Addis Ababa, Ethiopia. The plant received authentication from Botanist Reta Regassa, and a voucher specimen numbered BD00135/24 was submitted to the HU Herbarium at Hawassa University for future reference. An adequate quantity of plant samples will be gathered, and the freshly collected specimens will be subjected to three washing cycles in the laboratory, initially with tap water to remove dust and subsequently with distilled water for rinsing. Subsequently and dries in the shade.

Chemical and Material

Chemicals: distilled water, ethanol (80%), ethyl acetate (AR), NaOH, silica gel (mesh 60–120), petroleum ether(AR), n-hexane, naphthol, iodine, KI, HCl(35–37%), chloroform(AR), H₂SO₄ (98%), FeCl₃, methanol (80%).

Material: Materials used for this study were; polyethylene bag, axe, carton, round bottom flask, Erlenmeyer flasks, plaster, beaker, funnel, Watch man No ≠ 1 filter paper, iron stand with full setup, Spatula, pipettes, washing bottle, measuring cylinder, Separating funnel, test tube, Capillary tube, reagent

bottles, tong, pencil, spatula, Petri dishes, volumetric flask, dropper, stirrer and person protective equipment's, analytical balance, electrical mill, water bath shaker (SHZ-82), rotary-vapor (RS-100), heat gun, hot plate.

Extraction

The finely powdered leaves of *W. somnifera* was macerated in a 1:8 ratio (100 kg to 800 ml) with 80% aqueous ethanol for 36 hours, occasionally shaken in a water bath shaker (SHZ-82) at 22⁰C. Subsequently, after sufficient shaking, the ethanol crude solution was filtered and concentrated with a rotary evaporator (RS-100) at 40°C, yielding greenish ethanol crude. Ultimately, the extracts were dissolved in distilled water and then successively partitioned with n-hexane, ethylene acetate, and n-butanol. The two immiscible solvent components were separated in a different container with a separating funnel, and each fraction was concentrated using a rotary evaporator (RS-100) at 40°C.

Phytochemicals screening

Ethylene acetate crude, n-hexane, and n-butanol solutions were used to test the phytochemical components. Using standard procedure, this is done to determine whether secondary metabolites are present or absent ^{11,5}.

Test for alkaloids

Few drops of Mayer's reagent were added to 1ml of extract. A yellowish or white precipitate was formed, indicating the presence of alkaloids ⁵.

Test for flavonoids

Two to three drops of sodium hydroxide were added to 2 mL of extract. Initially, a deep yellow color appeared but it gradually became colorless by adding few drops of dilute HCL, indicating that flavonoids were present⁵.

Test for phenols

Two milliliters of 5% neutral ferric chloride solution were added to 1 ml of extract, the dark blue coloring indicating the presence of phenolic compounds ⁵.

Test for glycosides

Few drops of Molisch's reagent will be added to 2 ml of extract and will be shaken well. 2ml of conc. H₂SO₄ will be added on the sides of the test tube. A reddish violet ring appeared at the junction of two layers immediately indicated ⁵.

Test for terpenoids

About 5 mL of extract will be dissolved and mixed with 2 ml of chloroform, then 3 mL of concentrated H₂SO₄ were added and reddish brown coloration at the interface will confirm the presence of terpenoids ⁵.

Test for tannins

0.1% FeCl₃ solution added to few ml of extract and the blue, blue-black, green or blue-green color which confirmed the presence of tannins ⁵.

Test for saponins

A drop of Na₂CO₃ solution was added to 5 ml of extract in a test tube. After vigorous shaking, it was left to rest for five minutes. Foam formation indicated the presence of saponins ⁵.

Test for steroids

2 ml of chloroform and concentrated H₂SO₄ were added with the 5 ml aqueous plant crude extract. In the lower chloroform layer red color appeared that indicated the presence of steroids ⁵.

Anti-bacterial evaluation

Antibacterial assessment of the crude leaf extracts of *W. somnifera* was conducted at Madda Walabu University, Goba Referral Hospital, and the Microbiology Clinical Laboratory employing the paper disk diffusion technique.

Following the suggestions of the Clinical and Laboratories Standard Institute (CLSI, 2012), the antibacterial activity of the crude extract was assessed using the paper disc diffusion technique against two gram-positive bacteria (*S. aureus* and *S. pyogenes*) and two gram-negative bacteria (*E. coli* and *K. pneumonia*). With sterile forceps, each disk of Whatman filter paper Number 1 (6 mm in diameter) was meticulously placed into the agar and subsequently autoclaved for 15 minutes at 121°C for sterilization. Following the preparation of Muller Hinton agar and its placement onto sterile culture plates, the plates were dried for ten minutes. A sterile cotton swab was then used to transfer a bacterial culture onto the Muller Hinton agar plates. A sterile cotton swab was used to evenly spread the bacteria over the entire surface of Mueller Hinton agar, which was then utilized for the sensitivity test. Sterile filter paper discs, previously infused with a known concentration of plant extracts, were placed on the surface of Mueller Hinton agar. Bacterial strains were inoculated onto MHA plates, inverted, and incubated for 24 hours at 37°C. After incubation, the diameter of the zones of total inhibition, including the disk, was measured and documented in millimeters

Results and Discussion

Extraction yields

From the maceration of 500 g of *Withania somnifera* plant powder, 16.50 g of brown crude was obtained. The partitioning process of crude ethanol included dissolving the extracted crude in an aqueous solution, beginning with the least polar solvent (n-hexane), progressively increasing solvent polarity, and creating a separating layer because of the two solvents' immiscibility. After partitioning the ethanol crude into ethylene acetate and n-butanol fractions, a total of 7.5 g of yellowish ethylene acetate crude and 5.50 g of n-butanol crude were obtained. Nonetheless, assessing hexane is extremely difficult.

Partition of n-hexane

Crude ethanol extracts dissolve in water and are separated using n-hexane. A phase boundary is created because the two solvents do not mix.

The upper layer (grey color) floating above the aqueous solution (Fig. 1) is a crude n-hexane solution and is gently transferred to another container. The hexane solution is concentrated by being left at room temperature, resulting in only a small quantity of crude.

Partition of Ethylene acetate

Chloroform partitioned within the ethanol-water solution, creating a phase boundary where the yellowish solution floated above the aqueous layer.

The solution in the upper layer (yellowish) (Fig. 2) is ethylene acetate, and the layers are separated. The solution is ethylene acetate evaporated, yielding 7.5 g of collected crude. In this section, a notably high mass percentage of secondary metabolites were revealed.

Partition of n-butanol

Finally, the aqueous ethanol was partitioned using n-buthanol (Fig. 3), resulting in the collection of 5.50 g of crude.

The leftover aqueous solution was discarded due to primary metabolites. Ethanol solvents can extract from the least polar compounds to much more polar ones (primary metabolites). Consequently, partitioning is employed to eliminate these primary metabolites from the crude extract and to differentiate the spectrum of secondary metabolites acquired according to their polarities.

Phytochemical Screening

Phytochemical analysis of primary secondary metabolites including alkaloids, flavonoids, steroids, saponins, tannins, terpenoids, and phenols was conducted on n-hexane, ethylene acetate, and n-buthanol crude extracts. In the ethylene acetate partition, terpenoids, phenols, flavonoids, and steroids were detected, while glycosides and tannins were found in the n-butanol partition (Table 1).

Table 1
Table of Phytochemical Screening on leaves of *Withania somnifera*

s/no	components	n-hexane	ethylene acetate	n- buthanol
1	alkaloids	+	-	-
2	flavonoids	-	+	-
3	phenols	-	+	-
4	glycosides	-	-	+
5	terpenoids	-	+	-
6	saponins	-	-	-
7	tannins	-	+	+
8	steroids	-	+	-

In general, the extraction of the leaves parts of in this study revealed *Withania somnifera* secondary metabolites like alkaloids, flavonoids, phenols, glycosides, terpinoids, tannins, and steroids and the result similar within ⁴. In contrast, saponins are not present in any of the crude extracts, and presented components are indicated by the "+" sign, while "-" denotes the absence of a component (Table 1). Table 1 show that the sugar component (primary metabolites) remained in the aqueous solution while nearly all of the secondary metabolites were detected in both solvents (ethylene acetate and n-butanol partition). The reason for this is that highly polar chemicals are attracted to water as a solvent.

Anti- bacterial Evaluation

The antibacterial effects of the crude extract from leaves *W. somnifera* were assessed using ethanol crude extract and its fractions (ethylene acetate and n-buthanol) on four bacterial strains at the same concentrations of 100 mg/ml, 75 mg/ml, and 25 mg/ml. The chosen bacterial strains consisted of two gram-negative (*E. coli* and *K. pneumonia*) and two gram-positive (*S. aureus* and *S. pyogenes*), with pathogen sensitivity indicated in diameters. The antibacterial effect of the initial crude (ethanol extract) at every concentration shows the largest inhibition zone, while the inhibition zones of its fractions (ethylene acetate and n-butanol) display different results (Table 2) as illustrated below.

Table 2

Table of Antibacterial evaluation crude extract from leaves of against *W. somnifera* four different stain bacteria

Solvent	Sample in mg/ml	Types of bacteria with mean inhibition diameter (mm)			
		<i>S. aureus</i>	<i>S. progenies</i>	<i>E. coli</i>	<i>K. pneumonia</i>
Ethanol	100	19	17.5	15.5	14
	75	17	15.5	10.7	10
	25	14.5	8.5	6.5	8.5
ethylene acetate	100	18.6	15.8	14.6	13
	75	15.5	14.7	13.5	10.5
	25	11.5	10.5	7.4	6.7
ButOH	100	15.3	14.9	-	-
	75	10	9.3	-	-
	25	5	4	-	-
Cipro	12.5	26	25	20.5	18.5

According to Table 2, the n-butanol fraction shows invisible results at low concentrations, yielding the lowest outcomes across the entire spectrum. The actions of the crude fractions suggest that the ethylene acetate fraction effectively inhibits the growth of entire pathogens at all concentrations, likely due to the different chemical compounds found in the ethylene acetate crude fraction (Table 1). The inhibition was notable for *S. aureus* from gram-positive bacteria and for *E. coli* from gram-negative bacteria. The standard ciprofloxacin (12.5mg/ml) acted as the control for both bacterial strains and showed the greatest inhibition zone against all pathogens.

Conclusion

According to the results, 16.50 g of crude ethanol was derived from 500 g of macerated powdered stem bark of *W. somnifera*, which was then further fractioned into n-hexane, ethylene acetate (7.5 g), and ButOH (5.5 g). The results indicated that the ethylene acetate crude fraction from the leaves of *W. somnifera* showed a higher mass percentage compared to other solvent extracts. The study validated the existence of phenols, alkaloids, tannins, terpenoids, steroids, and glycosides in *W. somnifera*. For antibacterial tests, all the extracts exhibited strong antibacterial properties at high concentrations. Among the samples of crude fraction examined, the crude extract of ethylene acetate showed significant activity against the growth of all pathogens at every concentration. The crude extract was found to contain phytochemicals that contribute to antibacterial effects.

Abbreviations

BuOH	Buthanol
E. coil	Escherichia coli
EtOAc	Ethyl Acetate
MHA	Muller Hinton Agar
K. pneumonia	Klebsiella pneumonia
S. aureus	Staphylococcus aureus
S. pyogenes	Streptococcus pyogenes

Declarations

Data availability

The data and material used to support the findings of this study are available main body text and are additional available from the corresponding author upon request.

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Author contributions

Defar Bekele: conceptualization, writing (original draft), Conceived and designed the experiments methods, performed the experiments, analyzed and interpreted the data, contributed reagents, materials, analysis tools or data, Writing (review and editing), preparing figures, collecting data, validation investigation, and Formal analysis.

Mekonnen Abera: performed the experiments, analyzed and interpreted the data, contributed reagents, materials, analysis tools or data, Writing (review and editing), preparing figures, collecting data, validation investigation, and Formal analysis.

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Competing interests

The author declares no competing interests.

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Figures

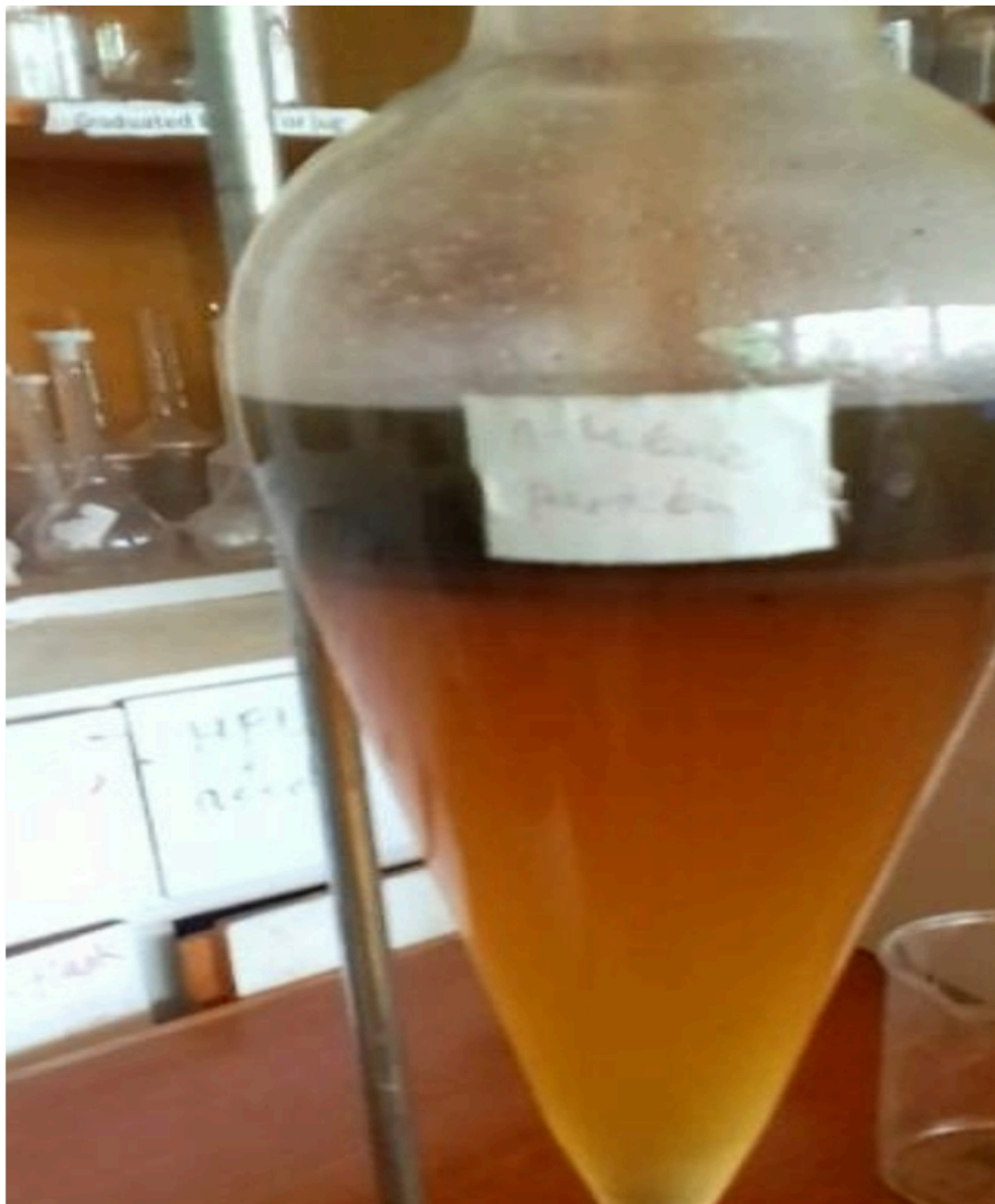


Figure 1

partition by hexane

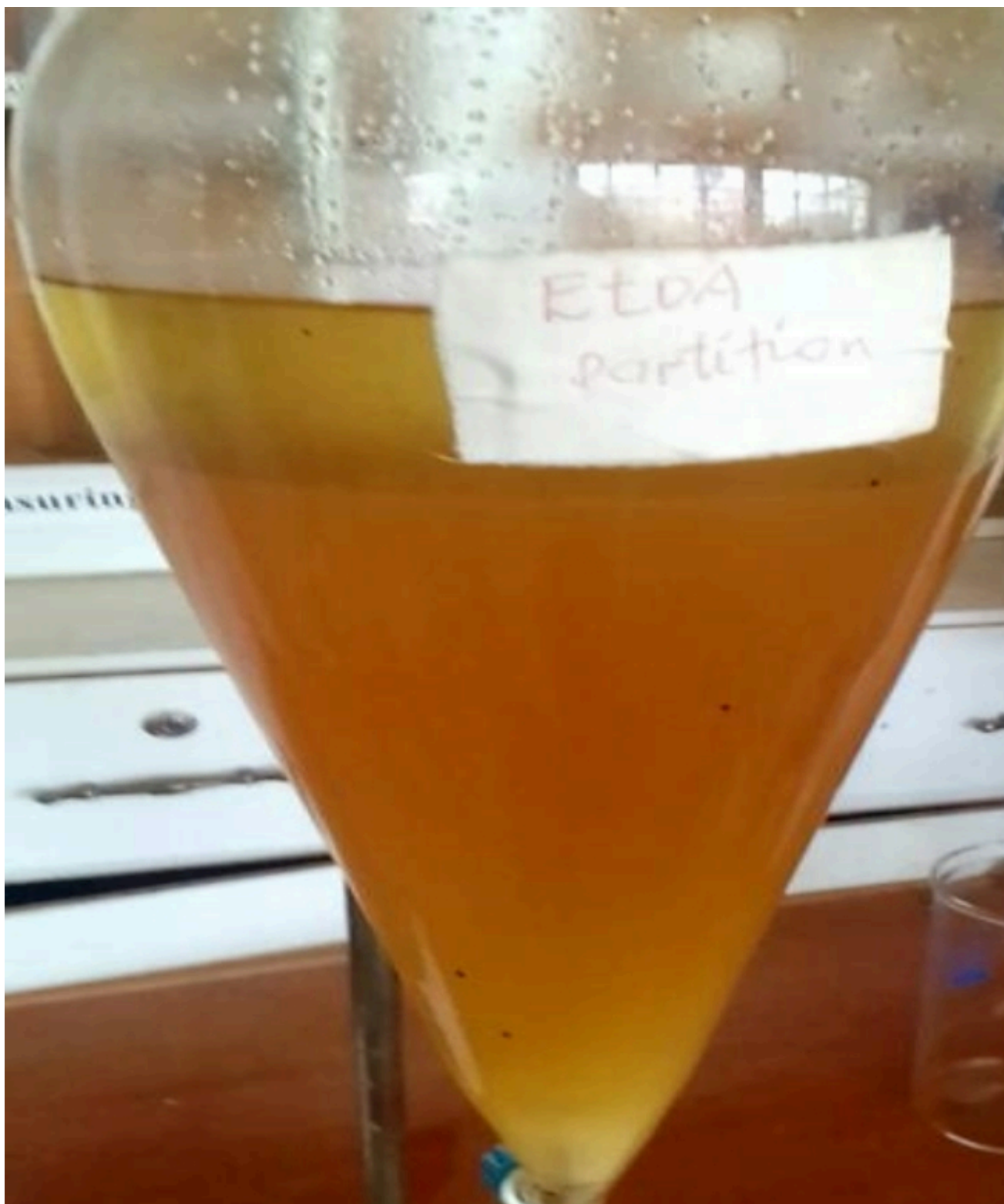


Figure 2

partition by ethylene acetate



Figure 3

partition by n-buthanol

Supplementary Files

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