IASR J. Med. Pharm. Sci.
ISSN:2582-9343
Website: https://ijmps.com/
Open Access Journal **Country**: India

IASR Journal of
Medical and
Pharmaceutical Science

ORIGINAL ARTICLE OPEN ACCESS

Phytochemical screening and evaluation of Antimicrobial and Antioxidant activity of Cannabis sativa leaves extract

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Received:24-04-2025
Accepted:05-05-2025
Availableonline:23-05-2025

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ABS TRAC T

Objectives: The study focuses on extracting and analyzing bioactive compounds from the plant's leaves, particularly cannabinoids and flavonoids, known for their antibacterial and antioxidant properties. By optimizing extraction methods and assessing these compounds' efficacy, the research aims to enhance the understanding of Cannabis sativa therapeutic potential in alternative medicine.

Methods: This study extracts bioactive compounds from Cannabis sativa using Soxhlet extraction with methanol and petroleum ether. The extracts are screened for phytochemicals and tested for antibacterial activity against Gram-positive and Gram-negative bacteria, as well as antioxidant activity using the DPPH assay, to assess its potential medicinal properties.

Result: Cannabis sativa leaves were extracted with methanol (20.76% yield) and petroleum ether (14.1% yield). The methanol extract showed higher antioxidant activity (IC50 = 98.1 µg/ml) compared to petroleum ether (IC50 = 106.43 µg/ml), with ascorbic acid showing the highest activity (IC50 = 16.2 µg/ml). The methanol extract also exhibited strong antibacterial effects, particularly against E. coli and Pseudomonas, with inhibition zones up to 20 mm. Phytochemical analysis revealed alkaloids, terpenoids, tannins, and glycosides in both extracts, with methanol also containing resins, suggesting it is more effective for extracting bioactive compounds from Cannabis sativa.

Conclusion: Cannabis sativa leaves exhibit notable antioxidant and antibacterial properties. The methanolic extract, with the highest antioxidant activity (IC50 = 98.1 µg/ml) and effective antibacterial action.

Keywords: Antibacterial activity, Antioxidant activity, Cannabis sativa, phytochemical screening, soxhlet extraction.

INTRODUCTION

Cannabis sativa is an annual plant in the Cannabaceae family. The Cannabis plant deeply rooted in Indian tradition named as Bhang which is often associated with Lord Shiva and considered holy by Hindus. The industry use of *Cannabis sativa* dates back to ancient china, where it was one of the oldest recorded pharmaceuticals used for treating ailments like rheumatic pain, constipation, female reproductive disorders and malaria. By the 19th century, its therapeutic potential had garnered attention in the western world, with the first clinical conference on *Cannabis sativa* held in 1860(1). *Cannabis sativa* is a medical plant that has attracted much attention for its possible therapeutic uses. This is primarily because its flowers and seeds are high in cannabinoids like CBD and tetrahydrocannabinol (THC). Numerous medical uses for these cannabinoids, such as pain management, anti- inflammatory properties, and neuroprotection, have been investigated in depth. According to Hanuš (2009), such an investigation can reveal new therapeutic compounds and enhance our understanding of the plant's complete medicinal range. Apart from their phytochemical component, Cannabis sativa leaves also possess antibacterial properties that have not been thoroughly

studied. With conventional antibiotics losing their effectiveness against some bacterial and fungal infections, antimicrobial resistance is a major worldwide health concern (World Health Organization [WHO], 2022). The varied chemical structures and methods of action of plant-based chemicals have made them attractive candidates for development as novel antibacterial agents. One significant gap in the field is the lack of thorough study on the phytochemical composition and antibacterial qualities of Cannabis sativa leaves. Closing this disparity will help us better understand the plant's therapeutic qualities and create useful applications that may improve public health(2).
Antibacterial agents are the substances that fight against pathogenic bacteria by killing the metabolic activity of the bacteria and inhibit the growth of bacteria. Due to their distinctive structure, Gram negative bacteria are more resistant than the Gram positive bacteria and cause a significant morbidity and motality over worldwide(3).
Antioxidants are compounds that prevent oxidation by neutralizing free radicals. Oxidation can create free radicals that initiate damaging chain reactions, leading to cell injury and death. Oxidative stress is a significant contributor to various chronic and degenerative diseases, including arthritis, diabetes, cardiovascular diseases, cancer, and atherosclerosis. Antioxidants help combat these effects by neutralizing free radicals and blocking oxidation reactions that drive disease progression. By inhibiting the formation of reactive oxygen species, antioxidants protect cells from oxidative damage. Flavonoids and phenolic compounds are key indicators of antioxidant activity. Their effectiveness is largely due to the hydroxyl groups in phenolic compounds, which donate hydrogen atoms through electron transfer to halt oxidation processes(4). Phytochemical screening helps in identifying the active compounds responsible for the plant's biological activity. The identification of effective antimicrobial compounds from Cannabis sativa leaves could lead to the development of new, plant-based treatments for bacterial and fungal infections. The results may lead to the identification of fresh antibacterial agents and bioactive chemicals, which would ultimately promote sustainable medical practices and result in more successful therapies.

METHODS AND MATERIALS

Study Design

This is an experimental design that Is being conducted to evaluate the antibacterial activity
, phytochemical screening and antioxidant activity.

Study site

The study site consists of a research laboratory on Hope International College microbiology lab and CNP LAB which is equipped with cutting-edge analytical and microbiological tools,and a microbiological testing facility for assessing antimicrobial activity. All of these facilities are done within the college with safety regulations.
Plant collection and identification

The plant of Cannabis sativa was collected from Gwarko, Lalitpur. The herbarium of whole Cannabis sativa plant was prepared and taken to National Department of herbarium for the identification in Godawari , Lalitpur.

Extraction process (5)

The leaves of Cannabis were cleaned and shade dried for almost 2 weeks . The dried leaves was then pulverized into coarse powdered form by using a mortar and pestel and stored properly in zip bag for further use.
The powder of Cannabis leaves ere extracted by gradient extraction in order to increase the polarity. The solvent used are Methanol and Petroleum ether. 30 gm of powder sample was placed in thimble and kept inside the Soxhlet apparatus. The sample were then successively extracted by using hot soxhlet extraction method in Pharmacognosy laboratory. The apparatus was run for 24 hr till the solvent appear in siphon that contain a crude extracts of the samples. After completion of extraction the solvents were evaporated in hot water bath at 60-70°C. The dried sample were then weight and sealed with aluminum foil and stored in desiccator for the further use.

Phytochemical studies

The dry extract from the soxhlet extraction method should be subjected for the phytochemical screening(6).

Antibacterial Activity (7)

Preparation and sterilization of growth media

Muller Hinton Agar (MHA):

The cultivation media for microbial growth was carried out by MHA .During this process 38gm of MHA was placed in 1000ml of conical flask and 1000ml Distilled water was added slowly with constant stirring for the dissolution and for the better dissolution heat was provided for better dissolution after complete dissolution the conical flask was plug with the cotton and aluminum.

Nutrient Broth:

28 gm. of nutrient broth was placed in 1000ml conical flask and 1000ml distilled water was added slowly with continuous string for the dissolution and for the better dissolution the solution was heated till the complete dissolution

after that the conical flask was plug with the cotton and aluminum.

These conical flask were placed into autoclave for sterilization at 15lbs for 15 min. After that the conical flask were placed sterilized laminar air flow and left for cooling.

Sterilization of petri dish plate

Sterilization of petric dish plate was carried out by the hot air oven at 160°C for 60 minutes by wrapping with newspaper. After sterilization, the disc were placed into laminar air flow.

Pouring of growth media

Growth media was poured properly in sterile petri disc plate in aseptic condition under the flame of burner and allowed for the solidification the plate were wrapped with paper tape and was stored in refrigerator for the further used.

Microorganism for Test

The bacteria stain used for the antibacterial test were provided from the National Pathology Lab (NPL). Two strains of bacteria were used, Gram positive bacteria staphylococcus aureus and Gram negative bacteria Escherichia coli, Klebsella and pseudomonas.

Antibacterial Test (8)

The available bacterial species was grown in the nutrient agar at 37°C and reactivated for the further use in nutrient broth. Prepared MHA plates were swabbed with culture of respective bacteria grown in nutrient broth. Extract were prepared at different concentration (50mg/ml, 100mg/ml, 200mg/ml, and 300mg/ml) in DMSO solution. Ciprofloxacin, azithromycin, amoxicillin was taken as standard. Both the extracts and the standard antibacterial drug were applied in the petri plates with bacteria. The plates were then incubated at 37°C for 24hAntibacterial test was evaluated by measuring the diameter of inhibition zone of the tested bacterial and the zone of inhibition by standard are also measured.
The inhibitory of DIZ was expressed in millimeter. After 24hr, the plates were examined for zone of inhibition.

Antioxidant activity (9)

DPPH Radical scavenging activity

Preparation of 0.1mm DPPH solution:

An accurately measured quantity of 0.00395gm DPPH was dissolved with methanol and diluted with methanol in 100ml volumetric flask.

Preparation of standard solution:

An accurately measured quantity of 0.25gm of standard Ascorbic acid was dissolved with methanol in 250ml volumetric flask and the volume was made with methanol.

Preparation of Test solution:

Stock solution with concentration 100mcg/ml was made by dissolving the 25 mg of each plant extract with methanol in 250 ml volumetric flask. The volume was made upto the mark with methanol. From stock solution different concentration (80mcg/ml, 60mcg/ml, 40mcg/ml and 20mcg/ml) of each plant extract for the test solution was made.

DPPH radical scavenging assay

4ml DPPH + 1 ml standard solution and prepared concentrated each test solution were mixed together in a test tube and incubated in a dark placed for about 30 minutes. The absorbance of each of the test sample and standard concentrations were measured at 517nm using UV -visible spectrophotometer.

4 ml DPPH + 1ml methanol was taken as a control.

DPPH Radical scavenging activity was calculated by using following formula:

DPPH Radical scavenging assay = Absorbance of control- Absorbance of test/standard
*100

RESULT

Extractive value

The leaves of Cannabis sativa were extracted using the different solvents like Methanol, Petroleum ether in order to perform and compare the phytochemical screening, antibacterialactivity.
The weight of extract obtained from Methanol and Petroleum ether was found to be 6.23
gm and 4.2g respectively.(Table 1 and Figure 1)

Table1: Extractive yield of leaves extract in different solvents

S.N	Solvent	Sample Weight (gm.)	Weight of extract (gm.)	Yield %
1.	Methanol	30gm	6.23 gm.	20.76
2.	Petroleum ether	30gm	4.2gm	14.1

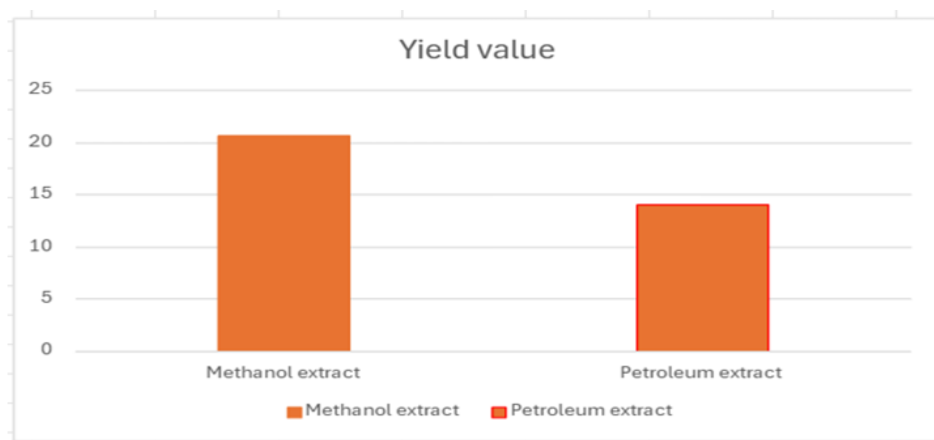


Figure 1: Percentage yield of the extract from the leaves of Cannabis sativa plant using different solvents

Phytochemical screening

The phytochemical screening of the methanol extract and petroleum ether extract was done to detect the secondary metabolites like alkaloid, steroid, falvonoids, terpenoids, tannin, carbohydrates, and saponins, glycoside, and resin by using standard phytochemical procedures. (Table 2)

Table2: Phytochemical screening of leave extract of Cannabis sativa

Table2: Phytochemical screening of leave extract of *Cannabis sativa*

S.N	Test	Methanol	Petroleum ether
1	Alkaloid Test		
	Mayer's test	+	+
	Wagner's test	+	+
	Hager's test	+	+
2.	Steroid Test		
	Salkowski Test	-	-
3.	Glycoside Test	+	+
4.	Flavonoid Test		
	a. Alkaline reagent test	-	-
	b. Shinods test	-	-
5	.Terpenoid test	+	+
6	.Saponin test	-	-
7	.Carbohydrates test		
	a. Molish test	-	-
	b. Fehling test	-	-
	C. Coumarin test	-	-
8	.Resin test	+	+
9	.Tannin Test	+	+

Antibacterial activity Test

The antibacterial activity of Cannabis sativa leaves extract were done by the disc diffusion method. (Table 3 to 5 and Figure 2 to 13)

Table3: Antibacterial activity of methanolic and pertroleum extracs of Cannabis

Name of Bacteria	Concentration (mg)	Zone of Inhibition (mm)	
		Methanol	Petroleum ether
E.coli	300	20	10
	200	15	5
	100	14	6
	50	13	4
S.aureus	300	14	11
	200	12	9
	100	9	7
	50	7	4
Klebsella	300	9	7
	200	7	9
	100	7	8
	50	2	5
Pseudomonas	300	15	10
	200	8	7
	100	9	6
	50	7	4

Table 4. Antibacterial activity of Azithromycin, Ciprofloxacin and amoxicillin as positive control in different bacteria

Name of Bacteria	Azithromycin	Ciprofloxacin	Amoxicillin
E.Coli	20	33	18
S.aureus	18	15	19
Klebsella	8	16	20
Pseudomonas	20	21	16

Table5: Antibacterial activity as DMSO as negative control in different bacteria

Name of bacteria	DMSO	Methanol	petroleum ether
E.coli	1	1	1
S.aureus	1	1	1
Kebesella	-	1	1
Pseudomonas	-	1	1

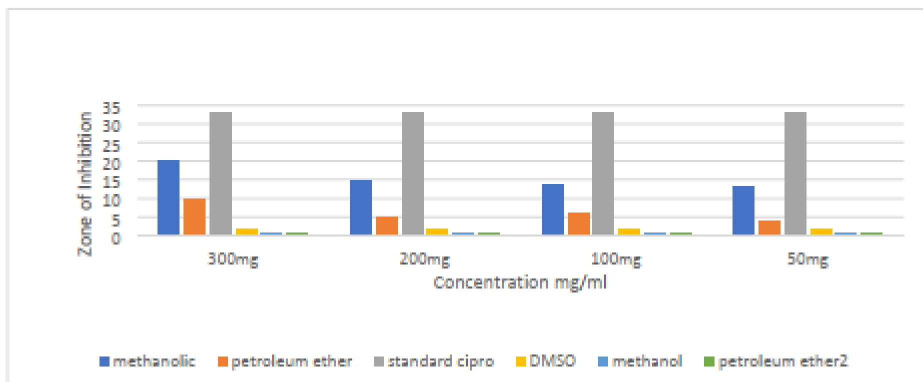


Figure 2: Antibacterial activity of different solvent extracts against E.coli

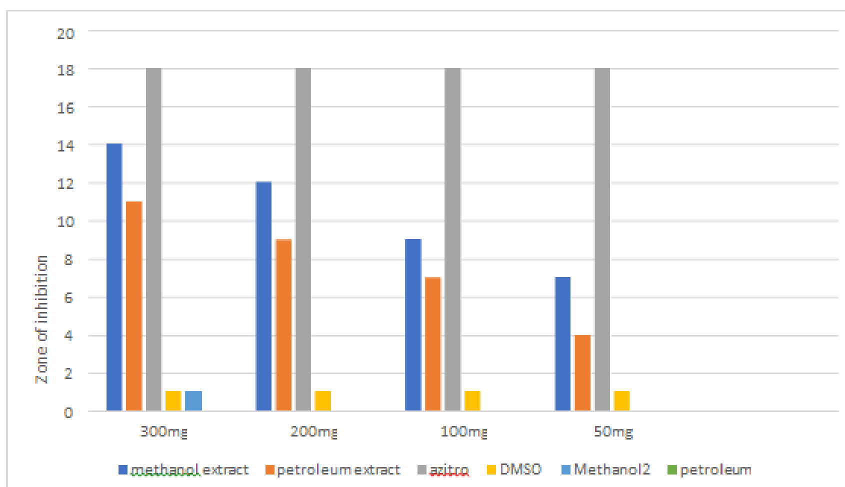


Figure 3: Antibacterial activity of different solvent extracts against *S.aureus*

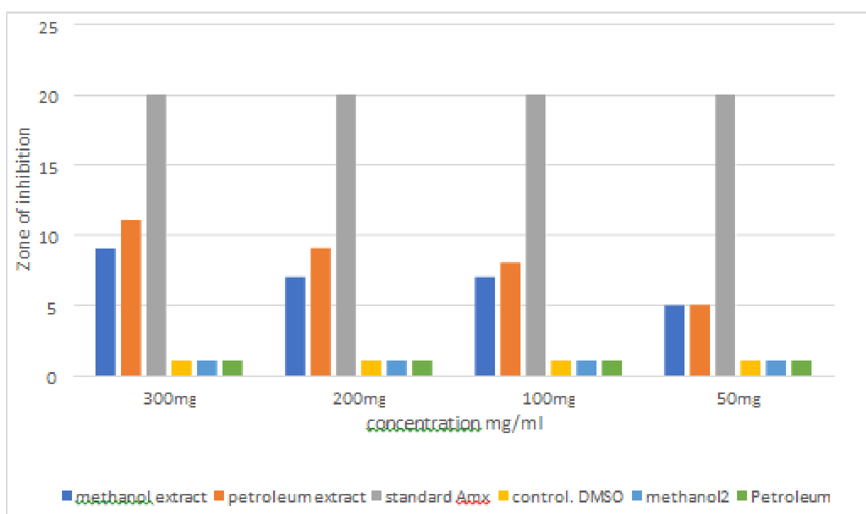


Figure 4: Antibacterial activity of different solvents extraction against *klebsella*

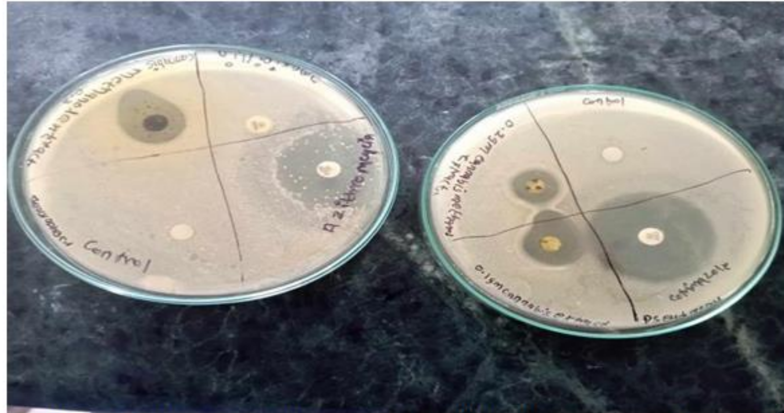


Figure 5: ZOI by methanolic extract with concentration (300mg/ml, 200mg/ml, and 100mg/ml against *Pseudomonas*

Figure 6: ZOI by petroleum ether extract with concentration 300mg/ml against *pseudomonas*

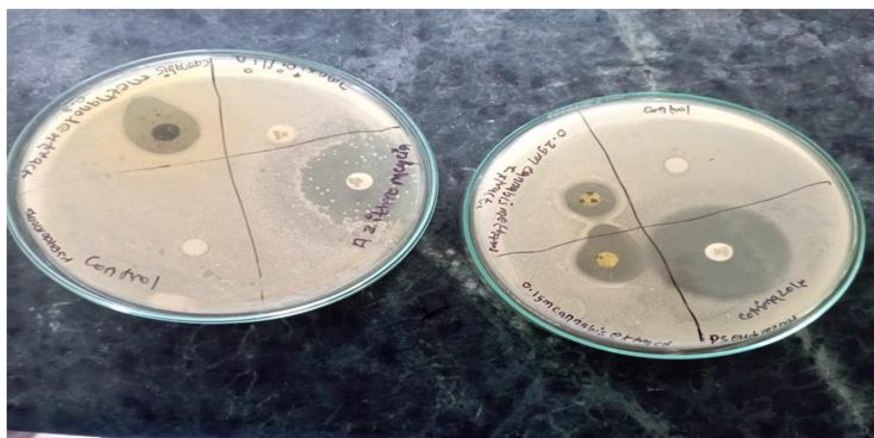


Figure 7: ZOI by petroleum ether extract having concentration 200mg/ml, 300mg/ml, 100mg/ml against *Pseudomonas*

Figure 8: ZOI by Methanolic extract having 300mg/ml and 200mg/ml against *Klebsella*

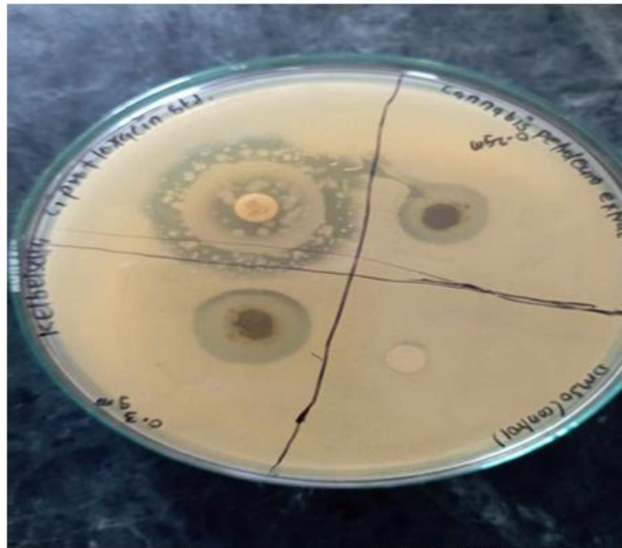


Figure 9: ZOI by petroleum ether extract having concentration 300mg/ml and 200mg/ml against *Klebsella*

Figure 10: ZOI by Methanolic extract having 300mg/ml and 200mg/ml against *E.coli*

Figure 11: ZOI by Methanolic and petroleum ether extract having 100mg/ml and 50mg/ml against *pseudomonas* and *Klebsella*

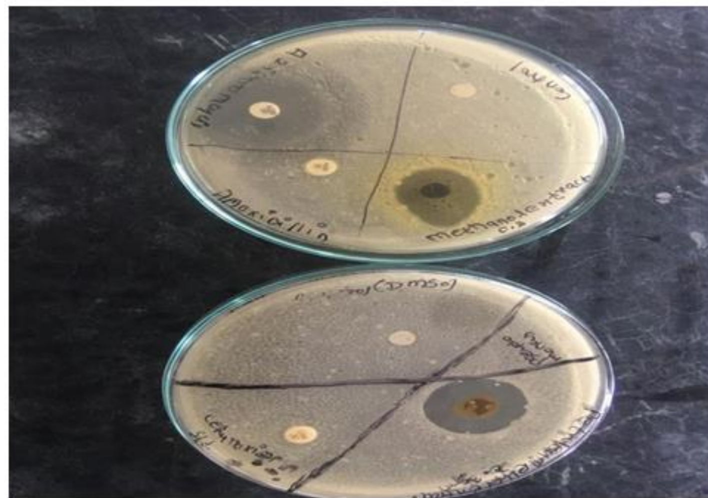


Figure 12: ZOI by Methanolic extract against S.Aureus

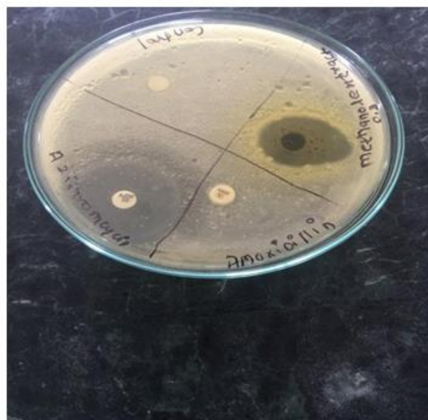


Figure 13: ZOI by Petroleum ether extract against *S.aureus*

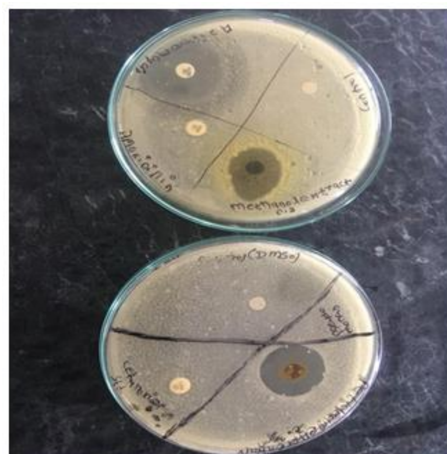


Figure 14: ZOI by petroleum ether and methanol extract against *E.COLI*

Antioxidant activity

The antioxidant property of Cannabis sativa leave extract was observed by DPPH free radical scavenging activity. DPPH free radical scavenging activity (IC50 value) of methanol extract of cannabis was evaluated to estimate their antioxidant activity. The asorbic acid used as standard, whereas DPPH free radical scavenging activity was determined on the basis of their concentration. (Table 6 and Figure 15 to 18)

Table 6: DPPH free radical scavenging assay

S.N	Concentration (ppm)	Scavenging value %		
		Methanol	Petroleum	Ascorbic acid
1.	20	14.55	13.5	48
2.	40	20.40	19.52	53.11
3.	60	35.49	28.63	59.04
4.	80	41.76	39.3	63.16
5.	100	49.52	47.25	68.34
	IC50= 98.1%	IC50=106.43%	IC50= 16.2%	

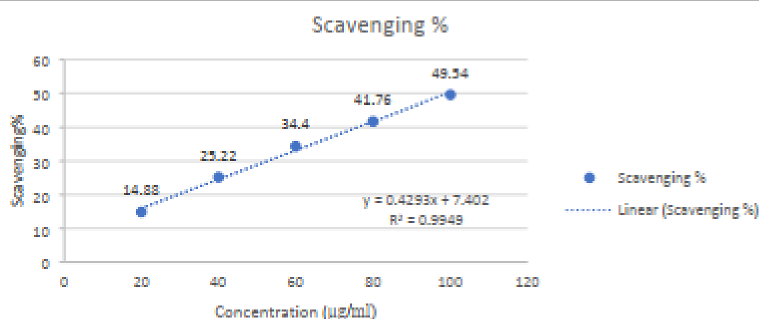


Figure 15: Correlation curve of scavenging activities % of methanol via DPPH

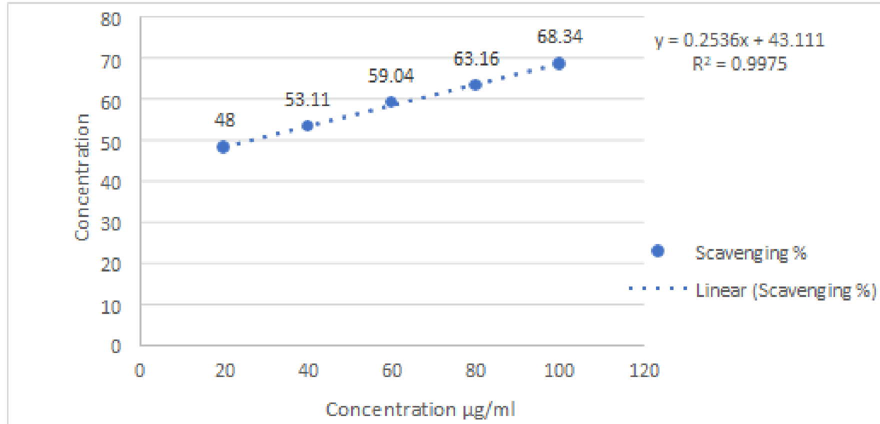


Figure 16: Correlation activity of scavenging activity % of standard Ascorbic acid via DPPH

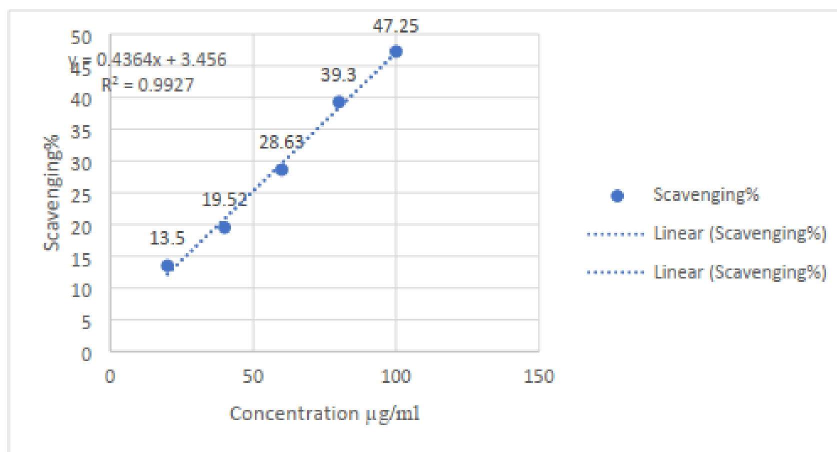
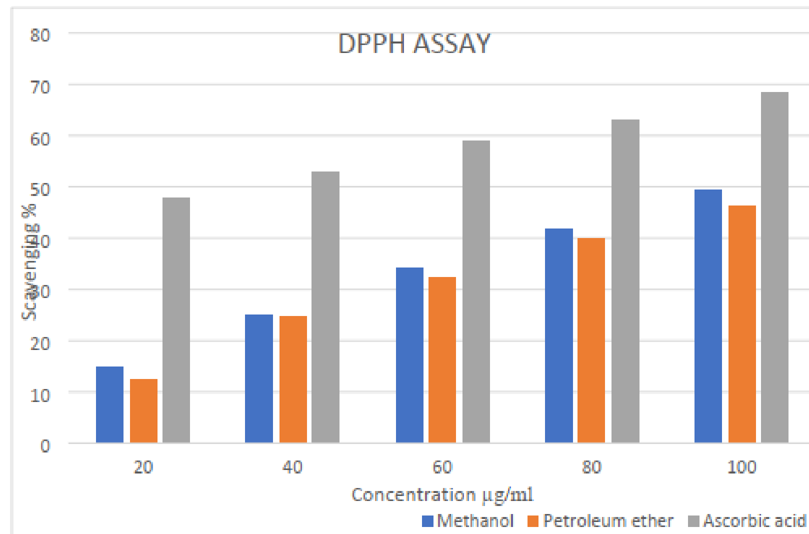


Figure 17: Correlation curve of Scavenging activity of petroleum ether via DPPH



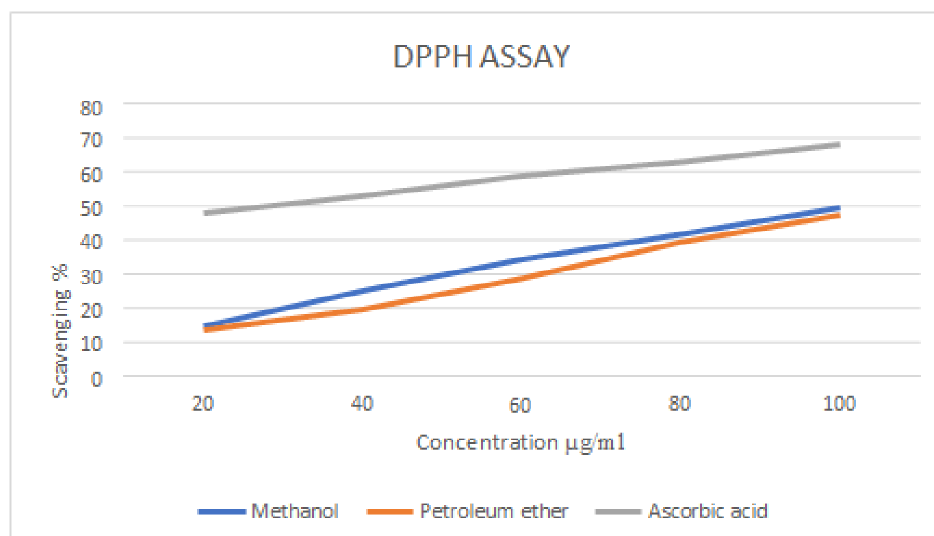


Figure 18: Scavenging % of Methanol, Petroleum ether extract with standard Ascorbic acid

DISCUSSION

Proximate analysis:

The % yield value of Cannabis sativa leaves of methanol and petroleum ether extracts was found to be 20.76 and 14.1 respectively. The % yield value of the obtained methanol extract is slightly greater to the result obtained by Anna stalsilowicz et al. and the yield value of petroleum ether is similar to the result of B.S Audu et al. by using petroleum ether solvent.

Phytochemical screening:

The phytochemical screening conducted by the B.S Auda et al. the Petroleum ether extracts of Cannabis sativa leaves confirms the presence of alkaloid, flavonoid, cardiac glycosides and terpenoids and absence of saponin, tannins, Phenol. Similarly in our study the alkaloids, Glycosides, Tannin, Terpenoids and resin are present whereas, Flavonoids, saponin, Steroid Carbohydrates are absent.

And the study done by the Sharma K et al. The methanolic extract of Cannabis leaves confirms the presence of Terpenoids, Tannins and carbohydrates. Similarly our study shows Terpenoids, Alkaloids, Tannins, resin and Glycosides are present. Whereas Steroids, Flavanoids,Saponin and Carbohydrates are absent.

Antibacterial activity:

The study of antibacterial activity from Cannabis leaves extract done by the Vinod Kumar et al. showed that the quit effective with 50mg concentration of methanolic extract against the Gram positive bacteria i.e. Staphylococcus aureus and pseudomonas but higher effective with concentration 200gm in Gram negative bacteria
i.e. E.coli, Klebsella. The ZOI in methanol extract shows 14 mm against the e.coli, staphylococcus aureus shows 12mm, Klebsiella10mm and pseudomonas 13mm.

From our study the ZOI showed that both extract of Cannabis sativa have a strong bacterial growth inhibitory activity against E.coli highest in methanol extract 20mm, where as in petroleum ether 10mm. The highest ZOI was shown at 300mg than that of other concentration.

The study conducted by Jain V , Choudhury et.al reveal there Was less ZOI observed in methanolic extract of Cannabis leaves against the Klebsella .while 12.5 mm ZOI was observed against S.aureus,11mm was observed in E.coli. Whereas in our study showed highest ZOI at 300mg and 200mg concentration of methanolic extract against the pseudomonas . The highest ZOI 15mm was shown at 300mg methanolic extract against the pseudomonas whereas lower ZOI shown at 100mg (9mm) and 50 mg (7mm)against the Pseudomonas and E.coli. The study revealed 11-14mm indicates the low sensitivity and 15-25 high sensitivity.

The study conducted by Gildea L , Ayariga et.al reveal that Cannabis sativa belonging to the family Cannabinaceae were screened for their antimicrobial activity against two Gram positive organisms (Bacillus subtilis, Staphylococcus aureus), two Gram negative organisms (Escherichia coli, Pseudomonas aeruginosa). The petroleum ether extract of the whole plant exhibited pronounced antibacterial activity (23 - 28 mm) against both Gram positive organisms, high activity (16 mm) against Escherichia coli and inactive against Pseudomonas aeruginosa. Whereas in our study petroleum ether extract

shows higher bacteria activity in pseudomonas and Ecoli at the concentration 300mg (11mm) and in E.coli petroleum ether shows 12mm at concentration 300mg.In our study highest activity was showns at methanolic extract against the E.coli and pseudomonas than that of Klebsella and S.aeurus.

Antioxidant activity:
The study done by Anna Stasilowicz et al, reported that the IC50 value of standard ascorbic acid was found to 35.11 mcg/ml using DPPH assay. Cannabis shows a good range of antioxidant potential. Cannabinoids such as Cannabidiol (CBD) and tetrahydrocannabinol (THC) have gained a significant attention for their diverse therapeutic potential, including their antioxidant effects. In his study they reported that the antioxidant effect in leaves shows a more potential than other parts of Cannabis due to the presence of Flavinoids and phenolic compounds.

Matthew Ojezela et al reported that IC50 of methanolic extract was highly efficacious than the petroleum extract and was highly efficacious as compared to the Ascorbic acid. This study reported that IC50 of methanol was 94.83 in comparable manner with ascorbic acid IC50 96.02.
From the study done by Drinić Z et. Al. the IC50 value is correlated with the drug potency
i.e. the amount of drug necessary to produce the effect the lower Ic50 value the more potent the drug.
According to, Ojezele MO IC50 of 10 -50µg/ml classified as strong antioxidant compound. Compound with IC50 of 50-100µg/ml classified as intermediate antioxidant activity whereas IC50 >100µg classified as weak antioxidant.
In our study, the methanolic extract of Cannabis at different concentration (20mcg/ml, 40mcg/ml, 60mcg/ml, 80mcg/ml and 100mcg/ml) showed an IC50 value of 99.2mcg/ml. The petroleum ether extract of Cannabis at different concentration (20mcg/ml, 40mcg/ml, 60mcg/ml, 80mcg/ml and 100mcg/ml) showed an IC50 value of 108 mcg/ml and that of Standard ascorbic acid showed IC50 value 16.2mcg/ml. The methanolic extract of Cannabis shows an intermediate antioxidant property while petroleum ether extract shows a weak antioxidant property.

CONCLUSION
The research concludes the presence of Alkaloids, Flavonoid, Trepenoids , Tannins and resigns in methanolic extract while steroids , glycosides and carbohydrates are absence. Similarly in petroleum ether extract Alkaloids, terpenoids , flavonoids , glycosides are present, whereas Tannin, Resins and carbohydrates are absence.

The different slovents shows a ZOI for all the tested bacteria at the range of 4-20mm. The antibacterial potency shows a different with different concentration of plant extact.Overall methanol extract showed a more sensitive with S.aureus and klebsella whereas petroleum extarct showed a more sensitivity with E.coli and klebsella. Overall the Cannabis sativa shows a high potential antibacterial activity with Gram negative bacteria (E.coli and pseudomonas).

The petroleum ether extract with concentration (20mcg/ml, 40mcg/ml, 60mcg/ml, 80mcg/ml and 100mcg/ml) showed a low potent antioxidant property. Similarly the methanolic extarct with different concentration (20mcg/ml, 40mcg/ml, 60mcg/ml,

80mcg/ml and 100mcg/ml) showed an intermediate antioxidant property. The inhibition % increases with increasing concentration.

The above results shows a different possibility for a use of plant as potential source of several bioactive natural products and can play a significant role in the development of drug from the natural products for reducing the antimicrobial resistance and antioxidants effect from the natural products.

Acknowledgments
We would like to thank the faculty and staff of the Department of Pharmacy, HOPE International College and Hospital Pvt. Ltd, Purbanchal University, Kathmandu, Nepal,for their support and contribution to this research.

Conflict of Interest
The authors declare that there are no conflicts of interest regarding the publication of this article.

Author Contribution:
Hemank KC conceptualized the study, contributed to the methodology, curated the data, and wrote the original draft of the manuscript. Sanjeev Gurung was responsible for data analysis, visualization, and reviewing the manuscript. Binita Joshi performed the literature review and edited the manuscript, while Kalpana Thakur handled data collection and contributed to manuscript preparation. Pratima Adhikari conducted the investigation, participated in fieldwork, and contributed to writing. Rekha Dahal developed the methodology and provided writing assistance. Vijaya Yadav contributed to the conceptualization and overall supervision of the project. Sarad Pudasaini supervised the work and revised the manuscript. Satnarayan Sah conducted statistical analysis and finalized the manuscript. Rashmi Shrestha

contributed to data analysis and provided critical review, and Bhumika Ale was involved in data collection and preparing supplementary materials for the study.

Funding: The authors declare that no funding was received for the research, authorship, or publication of this article.

LIST OF ABBREVIATION

1. ZOI (Zone of Inhibition)
2. CBD (Cannabidiol)
3. THC (Trahydrocannabinol)
4. UTI (Urinary Tract Infection)
5. DNA (Deoxyribonucleic acid)
6. DPPH (2,2-Diphenyl-1-Picrylhydrazy)
7. DMSO (Dimethyl sulfoxide)
8. µg (Microgram)
9. mg(Milligram)
10. ml (Milliliter)
11. H2SO4 (Sulphuric acid)
12. IC50 (Half Maximal Inhibitory Concentration)

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