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EXPLORING MEDICINAL PLANTS EXTRACTS FOR ANTIBACTERIAL AND ANTIPROLIFERATIVE PROPERTIES: A FOCUS ON CANNABIS SATIVA, FICUS CARICA AND DODONEA VISCOSA

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Abstract
The traditional use of *Cannabis sativa, Ficus carica* and *Dodonea viscosa* in local medications to offset the bacterial infections. This study analysed phytochemical composition of medicinal plants leaves extracts in aqueous, acetone and methanol solvent. Antibacterial activity of extracts was assayed against 6 bacterial species *Staphylococcus aureus, Bacillus cereus, Bacillus pumilus, Streptococcus spp., Salmonella typhi and Acetobacter spp.* through the disk diffusion method. Antibacterial potential of extracts is explainable on bases of phytochemical composition as ethanolic extract strongly inhibits bacterial growth. Minimum inhibitory concentration (MIC) performed for ethanolic extract of *Cannabis sativa* at various concentrations of dilutions. *In vitro* antiproliferative potential was determined by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] on HeLa cell lines. Confluent monolayers of cells were used to test the cytotoxicity ethanolic extracts. ELISA reader calculated optical densities at 570 nm to measure the cell survival percentage and $EC_{50.}$ A dose dependent curve was obtained by plotting concentration of dose and cell viability. These findings promote the discovery of active compounds that might serve as lead molecules in the creation of novel antibacterial and antiproliferative medications.

Keywords-Traditional use; Cannabis sativa; Ficus carica; Dodonea viscosa; Phytochemical composition; Antibacterial activit

1. Introduction

Medicinal plants received significant consideration in the perspective of therapeutic phytochemicals, phenolics and flavonoids leading to the development of novel drugs that reportedly have positive effects against microbes and cancer (Azwanida, 2015). These organic compounds alkaloids, tannins, steroids and carbohydrates have definite correlation on physiological actions and metabolism due to bioactive elements (Yadav & Agarwala, 2011). Several secondary metabolites have been identified in the *Cannabis sativa, Ficus carica* and *Dodonea viscosa,* all plants were collected from from

Khalora kalan, Sherwan Tehsil of District Abbottabad in Khyber-Pakhtunkhwa (KPK) province of Pakistan.The extraction process's ability in isolating physiologically active compounds from plant material is primarily reliant on the kind of solvent employed. Low toxicity, ease of evaporation at low heat, promotion of quick physiologic absorption of the extract, preservation action, and inability to cause the extract to complex or dissociate are all qualities of a good solvent in plant extractions.

The phytochemical screening of *C.sativa* revealed that leaves have the highest concentration of phytochemicals, followed by stem and root. Steroids, fixed oil, resins, alkaloids, terpenoids, flavonoids, tannin, proteins, and amino acids, as well as phenolics, glycosides, and saponins are some of these substances. Numerous phytochemicals showed variation in extracts made using various solvents (Choudhary *et al.,* 2014). At the right concentration of phenolic and flavonoid compounds, Ficus carica has strong antioxidant capacity to scavenge free radicals. Antioxidant properties and phenolic and flavonoid concentration are related. Thus, it is demonstrated how *Ficus carica* leaf extracts are screened for potential antioxidants as sources of medicines for various illnesses, particularly oxidative stress and malignancies (Ayoub *et al.,* 2019). Among herbalists, *D. viscosa* is a commonly used plant for treatment of gout, rheumatism, and fever in folk medicine. Its leaves are used to cure bone fractures and as an anti-inflammatory, ulcer-healing, antibacterial, and antifungal agent. In various extraction solvents phytochemical components varied, though cardiac glycosides were absent in all the fractions (Riaz *et al.,* 2012). *In vitro* antibacterial and antiproliferative activities of these medicinal plants were analyzed with the most effective fraction to understand the molecular reasoning and effectivity.

2. Materials and Methods

2.1 Sample collection

The healthy and fresh leaves of wild *C. sativa, F. carica* and *D. viscose were collected from* Sherwan Tehsil of District Abbottabad in Khyber-Pakhtunkhwa (KPK) province of Pakistan and placed in sterile polythene bags.

2.2 Preparation of plant extract

Leaves of *C. sativa, F. carica* and *D. viscose* were washed to avoid contamination. Samples of medicinal plants were completely dried under shade and grinded to obtain fine powder. Various solvents including water, ethanol and acetone were used to formulate the extract in the wide range of polarity on the pH 7. Aqueous plant extracts were prepared by adding 15g of plant powder into 150ml of distilled water and mechanical shaking of mixture for 24 hours. Samples were filtered through whatman filter No.1 and stored in jars (Al-Manhel and Niamah, 2015). Ethanol and acetone extractions were contrived by soxhlet extraction, 15 g of plant powder were transformed by 100ml of solutions. To eliminate the solvent contaminations the soxhlet extractions products were preceded in rotary evaporation at evaporation points of solvents 78ºC and 56ºC, respectively ethanol and acetone. Extracts were stored at 4°C (Packialakshmi and Sowndriya, 2019).

2.3 Phytochemical screening

Standard processes were used to conduct the phytochemical analysis of different extracts of *C. sativa, F. carica* and *D. viscose* leaves. Phytoconstituents like flavonoids, terpenoids, anthraquinone, saponins, phlobatannins, cardiac glycosides, as well as steroids were analysed by conducting the qualitative phytochemical tests (Edeoga *et al.*, 2005).

2.3.1 Test for flavonoids (Alkaline Reagent Test)

Extract of 1ml dissolved into 5 ml of diluted ammonia and a few drops of sulphuric acid. Colour changes have been observed in each extract sample, yellow coloration showed the presence of flavonoids (Odebiyi and Sofowora, 1978).

2.3.2 Test for saponins (Foam Test)

Extracts 5ml vigorously shaken in tight test tubes resulted in persistent frothing on the surface of each extract of plant samples. Frothing was mixed with a few drops of oil (saturated lipids) and an emulsion appeared, indicating the saponin's presence (Santhi and Sengottuvel, 2016).

2.3.3 Test for phlobatannins

In test tubes 1ml of each plant extracts were taken and then 1% diluted hydrochloric acid (HCL) was added in each test tube. Then, these test tubes were boiled in the hot water bath for a few minutes until red precipitates were deposited in the bottom of the test tubes which confirms the presence of phlobatannins in the plant leaves (Raphael, 2012).

2.3.4 Test for anthraquinone

Extracts of 2ml of each plant leaves were taken and then 1 ml of ammonia solution was added in each test tube. Rosset colour appeared in the result, which indicates the presence of anthraquinone in the plant leaves (Kardong *et al.,* 2013).

2.3.5 Test for terpenoids (Salkowski test)

Every 2.5 ml of extracts were transferred in the test tube and after that 1 ml of chloroform was mixed into it. Then, 1.5 conc. sulphuric acid was cautiously mixed for the layer formation. An interface of reddish-brown colour appeared which indicates that terpenoids are present (Sheel *et al.,* 2014).

2.3.6 Test for cardiac glycosides (Keller-Killani test)

Each extract of 5ml had been taken in the test tubes and then 2 ml of glacial acetic acid was added into each test tube accompanied by one drop of ferric chloride solution. And then 1ml of conc. H_2SO_4 was transferred into the above solution. Due to which an interface of brown ring appeared which confirms the presence of cardiac glycosides as this interface represents the deoxy-sugar attribute of cardenolides. Appearance of violet rings can also be observed below the brown ring. Whereas a ring of green colour in the layer of the acetic acid, progressively through the whole thin laye (Yahaya *et al.,* 2020).

2.3.7 Test for tannin

Aqueous extract sample 2ml was taken and ferric acid 1-2 drops mixed in it. Green to dark green change in colour indicates the presence of tannin (Penesyan *et al.,* 2021).

2.4 Antibacterial activity

Antibacterial efficiency of various extracts synthesised by *C. sativa, F. carica* and *D. viscose* leaves analysed through agar disk diffusion method. Bacterial strains were inoculated (a standardised inoculum of 1 to 2 CFU ml 0.5 McFarland Standard) on Mueller Hinton agar plates by cotton swab through 24 hour freshly prepared LB broth. The sterilised 6mm diameter disk of whatman filter paper no1 was used to place extract samples on plate (Minahil *et al*., 2023). A combination of various concentrations of 10ul, 20ul and 60ul for each plant extracts were applied. Standard antibiotic disks of amikacin 30 µg used as a positive control group. Bacterial inoculated plates were incubated 24 hours at the temperature of 37°C. Each plate was preceded in triplicate. Diameter of inhibition zones originated by plants extracting discs and control was measured on every plate (Girish and Satish, 2008).

2.5 Anti-proliferative activity and cytotoxicity assay

The antiproliferative activity of ethanolic extracts were determined with MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The MTT assay was performed by the use of a 96-well cell culture plate which is flat bottom, and HeLa cell lines confluent band was

implanted to different wells (Bouyahya *et al*., 2018). The two-fold serial dilutions 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12µg/ml, 6µg/ml, 3µg/ml and 1.5µg/ml of each extract were made from 2X stock solutions in cell culture media in triplicate manner for antiviral and cytotoxic evaluation. HeLa cell lines were cultured in the DMEM media augmented by the 20% of the foetal bovine system dissolving 1.2 g of powdered media in 100ml of double distilled water, 10% foetal bovine serum was added and filtered by 0.22µm syringe filter. HeLa cells were counted with the help of the Neubauer chamber (hemocytometer). A cover slip was fixed on the hemocytometer. The 50µl of cell suspension was mixed with an equal volume of Trypan blue dye to make 1ml. This mixture was transferred to the hemocytometer through a pipette and waited for 1-2 minutes. The suspension of HeLa cells and dye was moved by capillary action under cover slip. The hemocytometer was placed on stage of an inverted microscope and the number of live (unstained) and dead (stained) cells per millilitre was counted (Moradi and Alidadi, 2016). Percentage viability of HeLa cells was calculated by following formula

HeLa cells revived from frozen stocks decontaminated with 70% isopropyl alcohol and it was thawed at 37ºC in a water bath. Then suspension of HeLa cells from cryovial was shifted to a centrifuge tube having 10ml of cell culture media. It was centrifuged, supernatant was discarded and pellets of cells were resuspended by adding 5 ml of cell culture media and transferred to Carrel cell culture flask, incubated it for 5 days at 37ºC with 5% CO_2 incubator and were examined routinely to observe confluency. When cells in flask attained a confluency of 80-90% then they were subcultures. Detachment of cells from the flask was made possible by 0.25 % of EDTA (Trypsin) with repeated subculturing. All the suspension of cells were poured into a sterile conical centrifuge tube and centrifuged it at 25ºC for 5 minutes at 200 xg. The supernatant was discarded; pellets of HeLa cells were dispersed in 10 ml of culture media and incubated at 37ºC for 5 days with 5% CO_2. By inverted microscope, cells were monitored in 24 hours (Talib and Mahasneh, 2010).

2.5.1 Treatment of HeLa cell line with medicinal plants extracts

Cells were seeded in 96- wells plate; the 100µl of cell suspension was poured under aseptic conditions in the safety cabinet. These plates were incubated with a specification of 37ºC temperature, 5% CO_2 for 5 days. Confluent monolayers of cells were selected for cytotoxic evaluation of ethanolic extracts of leaves. In each well 100µl of ethanolic extract was poured with multichannel micropipette. After 2-3 days of incubation cell culture media was removed thoroughly and phosphate buffer solution (PBS) was used for washing purposes (Kimani *et al.,* 2018). Then 100µl of MTT dye i-e 5mg/ml was added to each well and incubated for 3 hours. Stock solutions of plant extracts were prepared by dissolving the extracts in DMSO at a concentration of 20 mg/ml. Than dilutions of extracts were prepared in DMEM solution with final concentration of 1mg/ml (pre-treatment medium) and for 48 hours placed in incubator feasibility of HeLa cells were calculated by using 20 µl (50 mg/10 mL) of MTT dye solution in each well and providing a 3-hour incubation time. To lyse the cells DMSO was used as well as ELISA reader calculated optical densities at 570 nm (Tauchen *et al.,* 2015). The cell survival percentage (CSP) for both antiviral and cytotoxic activity was obtained by the following formula.Cell survival percentage= Mean OD of test – Mean OD of negative control × 100

3. RESULTS

3.1 Percentage yield of medicinal plant extracts

Weight of ethanol, acetone and aqueous extract was measured and the percentage yield for each sample was calculated.

Table 3.1: Percentage yield (%) of extracts

Botanical Name	Local Name	Plant part Used	Solvent	Percentage Yield (%)
Cannabis sativa	Bhang	Leaves	Ethanol	0.3
			Acetone	0.5
			Aqueous	0.27
Ficus carica	Fig	Leaves	Ethanol	0.8
			Acetone	0.9
			Aqueous	1.2
Dodonaea viscosa	Dhana Sar	Leaves	Ethanol	1.5
			Acetone	1.8
			Aqueous	2.4

3.2 Phytochemical analysis

Phytochemical screening of various extracts (ethanolic, acetone and aqueous) was performed to determine the presence of bioactive components like flavonoids, saponins, terpenoids, tannins, phlobatannins, anthraquinone and cardiac glycosides. Alkaloids are poisonous to microbes and have antiproliferative activities, all the extracts of three plants exhibited the presence of alkaloids. Steroids and anthraquinone are strongly related to interpret the biological process as anti-inflammation (Hajra *et al*., 2023). *C. sativa* acetonic extract deprived of tested metabolites through flavonoids were detected, on other hand aqueous and ethanolic extracts were of same phytochemical composition. In case of *F. carica* different extracts showed the same result except a little fluctuation of anthraquinone absences in acetone extracted samples. *D. viscosa* ethanolic extract samples have positive test results for all of the secondary metabolites, aqueous deprive anthraquinone while acetone has been positive for anthraquinone and flavonoids only (Raazia *et al*., 2023).

3.3 Antibacterial activities of medicinal plants

Antibacterial potential of medicinal plants was analysed through agar disk diffusion method. A standard antibiotic amikacin 30µlwas applied on plates as a positive control, in comparison all the extracts of plants were applied. After 24 hours of incubation, zones of inhibition were recorded for each extract in mm. *C. sativa* ethanolic extracts produced the maximum inhibition while the minimum zones were from aqueous extract. Mild antibacterial activity (<15mm) was shown by acetone extract of *C. sativa* leaves against *Streptococcus spp., Bacillus cereus, Staphylococcus aureus* and *Bacillus pumilus*, though moderate activity (16mm) was shown against *Salmonella typhi. Ficus carica* and *Dodonaea viscosa* acetone extract inhibition zones were much larger than ethanolic and aqueous extract. The maximum zone of inhibition was 32 and 24 respectively shown by acetone extract of leaves against *Bacillus pumilus.* These plant extracts exhibit antibacterial activity against gram positive and negative with effective inhibition zones. *Bacillus pumilus* and *Salmonella typhi* have been more affected by medicinal plants (Usman *et al*., 2023).

Table 3.2: Phytochemical of medicinal plants

Composition Identification Tests	***Cannabis sativa***			***Ficus carica***			***Dodonaea viscosa***		
Flavonoids (Alkaline Reagent Test)	**Aqueous**	**Ethanol**	**Acetone**	**Aqueous**	**Ethanol**	**Acetone**	**Aqueous**	**Ethanol**	**Acetone**
	+	+	+	+	+	+	+	+	+
Saponins (Foam Test)	+	+	—	—	—	—	+	+	—
Phlobatannins	—	—	—	+	+	+	+	+	—
Anthraquinone	+	+	—	+	+	—	—	+	+
Terpenoids (Salkowski test)	—	—	—	—	—	+	+	+	—
Cardiac glycosides (Keller-Killani test)	+	+	+	—	+	—	—	—	—
Tannins	+	+	+	+	+	+	+	_	_

Table 3.3: Medicinal plant extracts inhibition zones

Zone of inhibition (mm)										
	Control	***Cannabis sativa***			***Ficus carica***			***Dodonaea viscosa***		
Microorganism	**Amikacin**	**Ethanol**	**Acetone**	**Aqueous**	**Ethanol**	**Acetone**	**Aqueous**	**Ethanol**	**Acetone**	**Aqueous**
Staphylococcus aureus	24	22	8	6	21	13	_	18	16	13
Bacillus cereus	22	19	9	7	19	8	6	10	8	_
Bacillus pumilus	22	20	8	6	22	10	_	20	14	12
	21	20	12	7	20	7	_	11	10	7
Salmonella typhi	28	25	16	12	16	13	_	10	7	6
Acetobacter spp.	_	9	7	_	21	9	_	16	12	_

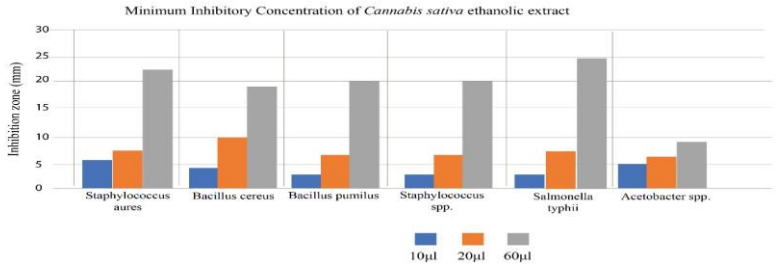

Fig 3.1: Minimum inhibition concentration of *Cannabis sativa* ethanol extract

3.4 Anti-proliferative activity

The antiproliferative activity of ethanolic extracts of medicinal plant leaves were evaluated against HeLa cell line with optical density using multi-well ELISA reader at 570 nm filters. Antiproliferative activity of ethanolic extract of medicinal plantleaves was determined at the various concentrations to estimate EC_{50} against HeLa cell lines. The effective concentration of 3.587 µg/ml, 41.6303 µg/ml and 46.4055 ug/ml respectively for *C. sativa,F. carica* and *D. viscosa* were calculated at which half number of cells are inhibited and half number of cells are alive. Graphs of cell viability plotted against the concentration of applied plant extract that predicted the improvement in treated cells with increase of dose.

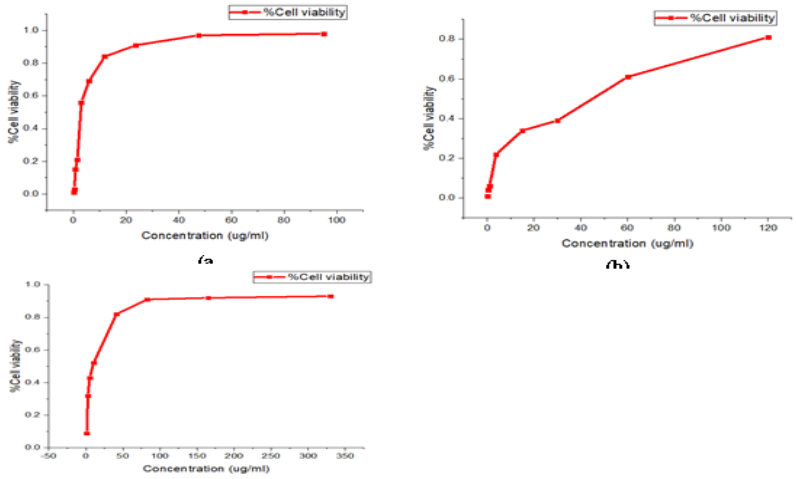

Fig 3.2: Cell viability percentage at various concentrations of ethanolic extract

Table 3.4 Cell survival fraction after dosing

Medicinal plants	**EC_{50} (ug/ml)**	**Survival fraction (%)**
Cannabis sativa	3.58	0.97
Ficus carica	41.6	0.5
Dodonaea viscosa	46.4	0.75

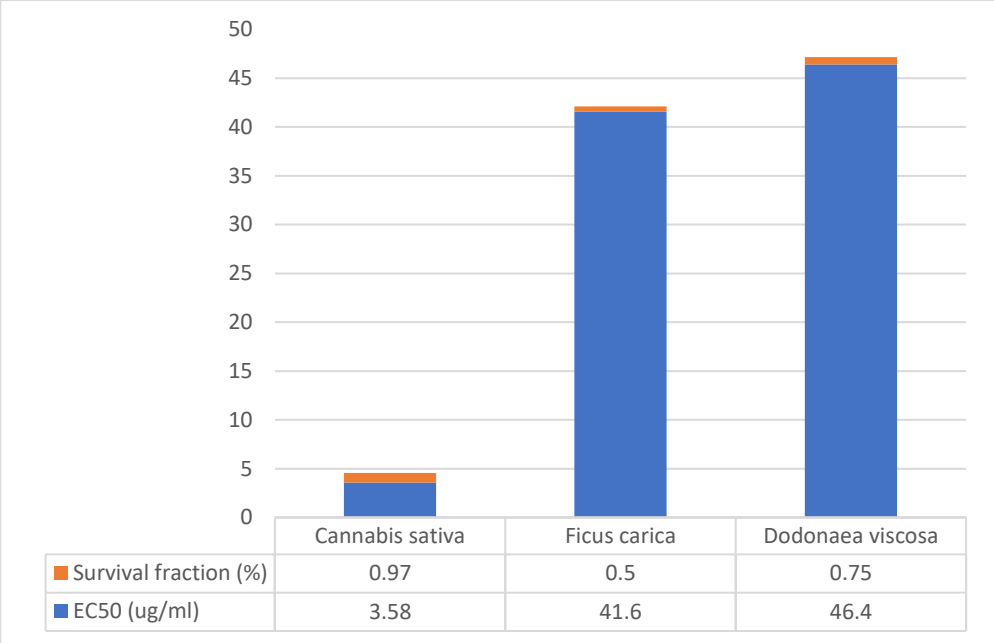

Figure 3.3. Cell survival and cell death percentage after dosing *Cannabis sativa, Ficus carica* and *Dodonaea viscosa* ethanolic extracts on HeLa cell line to calculate EC50.

Table: 3.5 Comparison of anti-proliferation efficiency of *Cannabis sativa, Ficus carica, Dodonaea viscosa*

Cannabis sativa			**Ficus carica**			**Dodonaea viscosa**		
EC50 = 3.58			EC50 =41.6			EC50 =46.4		
Concentration (µl/ml)	(CSP)	(CDP)	Concentration (µl/ml)	(CSP)	(CDP)	Concentration (µl/ml)	(CSP)	(CDP)
95	1.45	98.5	120	18.04	81.95	130	45	55
47.5	6.56	_	60	38.73	61.26	82.1	68	32
23.75	8.03	91.9	30	60.01	39.99	65.5	91	9
11.87	15.3	84.6	15	65.75	34.25	41.25	68	32
5.93	30.6	69.3	7.5	76.64	23.35	20.62	51	49
32.96	43.4	56.6	3.75	77.94	22.05	10.31	8	92
1.48	78.4	21.5	1.875	95.41	4.592	5.15	18	82
0.74	84.6	15.3	0.9375	82.85	17.14	2.57	9	91
0.37	96.3	3.65	0.4685	93.97	6.026	1.28	7	93
0.18	95.9	4.01	0.2344	98.17	1.829	0.64	14	86

4. Discussion

In the study local regional herbs were collected and experimented for antibacterial and antiproliferative activities that resulted in strong effectivity against bacteria and HeLa cell lines. Developing countries have pharmaceutical roots associated with use of medicinal plants, due to cost effective and lighter side effects (Ashraf *et al.*, 2020). The idea of synthesising drugs by using the natural resources emerged by phytochemical screening, rich ingredients of metabolites that directly affect the metabolism of living cells (Mahmood *et al.*, 2019). The maximum percentage yield of

plant samples from aqueous extract on polarity base solvents followed by acetone and ethanol (Abubakar *et al.,* 2020). Phytochemical screening of *Cannabis sativa,Ficus carica and Dodonaea viscosa* in ethanol, acetone and aqueous extracts gave the comparative results (table 4.2) through qualitative methods. Relevant reported literature predicted the presence of flavonoids, terpenes, saponin and anthraquinone in *C. sativa* (Ahmed *et al.,* 2019)a quite similar secondary metabolites anthraquinone and phlobatannins in *F. carica (*Abdel-Aty *et al.,* 2019*)* although an additional constituent cardiac glycosides and terpenoids in *D. viscosa (*Riaz *et al.,* 2012*).*

Presence of phenolic compounds in plants laid the foundation of antibacterial behaviour, as phenolic compounds free radical scavengers bind with the membrane of microbes to proceed the invading and result in alteration of the system. Cardiotonic effect is related to steroids and terpenoids produced by herbs that possess strong antiseptic and antibacterial potential. Tannins make the nutritional protein unavailable to microbes and competitive bulk of cells in case of proliferation. Cardiac glycosides inhibit Na+/K+ pump to store large amounts of Ca^{+} to restore the muscles contraction and relaxation, improving the cardiac distension. The inhibitory effect of aqueous and polar solvents of medicinal plant leaves were tested on enteric pathogenic and oral infection causing bacteria's *Staphylococcus aureus, Bacillus cereus, Bacillus pumilus, Streptococcus spp, Salmonella typhi and Acetobacter spp.* Ethanolic extracts of *C. sativa* produced clear and bigger inhibition zones in preliminary screening and proceeded for Minimum inhibitory concentration (MIC).On the other hand, acetone extracted samples of *F. carica* and *D. viscosa* have a better impact on growth inhibition of bacteria compared to ethanol and aqueous. The acidic fraction by ethanolic extract in *C. sativa* leaves turn it into a vulnerable antibacterial against both Gram positive and negative bacteria's, its due to cannabinoids prenyl moiety.

Terpenes of cannabis destroy the cell membrane permeability resulting from the leakage of intracellular components. The molecular mechanism behind the antibacterial activity of cannabinoids, is on structural modification level. There are five major types of cannabinoids that exhibit strong antibacterial potential with the MIC of 0.5ug/ml, ethanolic bonding with the carboxylic group of molecules introduce the prenyl moiety in cannabinoids. *F. carica and D. viscosa* extracts antibacterial behaved in agreement to literature as flavonoids hold the ability to inhibit nucleic acid synthesis, breaking down the energy metabolism and causing disruptions in cytoplasmic membrane. Liposomes of bacterial membranes leak out from intra liposomal spaces aggregates to block the transport across membranes. Other metabolites of fig leaves like tannins and terpenoid also participate by reacting with phospholipids and lipophilic compounds. MIC for ethanolic extract of *C. sativa* is 10ul, on this concentration all microbe's growth inhibits.

In-vitro anti-proliferation of plants were examined on the HeLa cell line by ethanolic extracts. The potential ability was assessed through OD at 570nm for 24 to 48 hours by recording cell viability. Antiproliferative and cytotoxic behaviours of collected plants were reported that fall in accordance with results. *C. sativa* separated cannabinoid types were applied on different cancer cells of humans (Anceschi *et al.,* 2022), isolated proteins from leaves and seed intrinsicate mitochondrial pathway under regulation of pro- apoptosis (Moccia *et al.,* 2020). Leavelatex study of F. carica proved the anticancer ability through DNA damage kit molecular studies of genotoxic and cytotoxic approved affectivity against MDA-MB-231 cells compared to the untreated control (AlGhalban *et al.,* 2021). *D. viscosa* leaves, bark and stem assayed against African green monkey (E6 vero), human prostate cancer cell line (DU 145) and breast cancer cell line (HCC 1395), most effective ones were leaves (Kaigongi, 2020).

Preliminary screening of antiproliferative activities through MTT assay effectively witnessed decrease in cell viability after increase of dose concentration in dose- dependent curve due to induced apoptosis. Ethanolic extracts of medicinal plants were moderately effective against cancer

line as calculated reading of EC_{50} *C. sativa, F. carica and D. viscosa were* 3.58µl/ml,41.6303 µg/ml and 46.4055µl/ml respectively. Phase contrast after various doses anticipated reduction in cell number as compared to applied control group DMSO. It is related to physiological changes in the cell from shrinkage to roundness.

5. Conclusion

It concluded that against pathogenic microorganisms, plants have strong defence compounds. The results show that plants play a part in an informal medical specialty. As it approaches the maturity of novel medications, which are needed today, the potential for producing anti-bacterial and anti-proliferation from leaves of plants appears satisfied. By removing the active components from plants, there are many opportunities to use plants in novel medications to treat a variety of disorders. As ethanol extracted samples showed good results in comparison to other extracts, more research is required to identify the active chemicals inside these plants.

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