

Antimicrobial profiling and synergistic interaction between leaves extracts of *Cryptolepis buchanani* (Roem. and Schult.)

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ABSTRACT

The following study explores the antimicrobial activity, synergistic effect, potentiation effect and phytochemical analysis of different extracts of *C.buchanani* leaves. *C.buchanani* leaves were dried and extracted by soxhlet extraction using four different solvents viz Ethyl acetate, Methanol, Acetone and Ethanol. The extracts of *C.buchanani* leaves were screened against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Xanthomonas citri*, *Citrobacter divergence*, *Vibrio cholerae*, *Candida albicans* & *Aspergillus niger*. The antimicrobial activity indicated that ethyl acetate extract gave maximum inhibition followed by methanol, acetone and ethanol. *Proteus vulgaris* was found to be most susceptible while *Aspergillus niger* was resistant to all the organic extracts for antimicrobial and synergistic activity. The combinations of ethyl acetate extracts has shown highest synergistic effect against *P.vulgaris*. Antagonistic effect was seen for fungal pathogens. Potentiation effect of Gentamicin was seen in ethyl acetate extract against *P.vulgaris*. The phytochemical constituents varied significantly depending upon the type of solvent used. Saponins and alkaloids were present in all extracts. Thus, *C.buchanani* is highly rich in the diversity of phytochemical constituents.

Key words: *C. buchanani*, Antimicrobial activity, Synergistic effect, Potentiation effect, Phytochemicals.

INTRODUCTION

Medicinal plants are referred to plants that are used for their therapeutic and medicinal values. Such plants provide basic raw materials for different industries such as pharmaceutical, cosmetic, perfumery, food etc. They are the major source of biodynamic compounds of therapeutic values. As there is an increasing demand for medicinal plants, investigations are being made on many known and unknown medicinal plants. For centuries, man has effectively used various components of plants or their extracts for the treatment of many diseases, including bacterial infections. Plants produce a wide variety of phytochemical constituents²¹, which are secondary metabolites and are used either directly or indirectly in the pharmaceutical industry. All the plant parts have medicinal properties, some act as antimicrobial agents. The determination of antimicrobial activities of different medicinal plants is of special interest these days due to the current global issue of increasing antibiotic resistance of microorganisms.

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Medicinal plants are significant source for obtaining drugs. India is blessed when it comes to vast diversity of flora. There are numerous plants with medicinal properties and study is going on such plants. In recent years antimicrobial properties of herbs are increasingly reported from different parts of the world.

Among many plants, one such less known plant is *Cryptolepis buchanani* Roem. & Schult which belongs to Periplocoideae subfamily of Asclepiadaceae^{11,15} and is commonly distributed throughout India, mostly in hot deciduous forests. It is woody twiners found in Eastern Ghats and is widely used as demulcent, diaphoretic, diuretic and cure for paralysis. *C.buchanani* is shade loving and found as a climber on some selected plant species such as “soti” (*Alstonia scholaris*). Sometimes, it is also found as creeper on ground. The leaves are green in color, 3-6cm in length, elliptical, smooth and shiny. They are dorsoventral and petiolate. The apex of leaves is mucronate and margin is entire¹⁴. The local names of the plant are, Hindi: Karanta, Dudhi, Shyamlatra, Gopavadhu, Kaalghatika; Bengali: Kalasariba; Sanskrit: Jambupatra sariva or Krishna sariva⁶.

Cryptolepis buchanani has been reported in Ayurveda for its uses as anti diarrhoeal, antibacterial, anti ulcerative, anti inflammatory, blood purifiers, cough treatment and lactation in women^{5,8}. It is of great medicinal value in treating arthritis⁹. Traditionally the plant, mainly its roots, stems and leaves are used for the treatment of bone fracture by tribal people¹⁸. Thus, the traditional use of this plant by tribal people for treatment of bone fracture indicates effective medicinal properties of the plant. Root extracts of *C.buchanani* has immunopotentiating properties and thus strengthen the immune system⁵. A chemical component named Nicotinoyl glucoside was reported in *C. buchanani*². Also, a new cardenolide named Cryptosin was isolated from the leaves of *C.buchanani*¹⁹. 2-hydroxy-4-methoxy benzaldehyde isolated from *C.buchanani* was found to be enhancing the cell to cell migration ability on wound healing assay⁷. Latex of plant is applied on the wound affected part externally¹³. *C. buchanani* shows the analgesic, anti-inflammatory and chondroprotective activities while it has no toxicity in cartilage explants. Therefore, *C. buchanani* may be useful as an alternative for the treatment of osteoarthritis⁴. Ethanolic leaf extract of *C.buchanani* significantly protects against liver injuries as well as oxidative stress¹⁰. The presence of phytoconstituents namely saponins, alkaloids and tannins in solvent extracts may be responsible for antifungal activity and thus *C. buchanani* has anti dermatophyte activity²⁰. We present the data on antimicrobial activity of four different extracts of *C.buchanani*. The efficacy of ethyl acetate, methanol, acetone and ethanol plant extracts against eight bacterial and two fungal species is described.

MATERIALS AND METHODS

Collection of plant leaves:

C.buchanani leaves were collected from forest of Ganeshgaon, Trimbakeshwar, Nashik, Maharashtra, India. The leaves were then washed with water and spread on filter paper to remove excess water and then were shade dried. The leaves were then grinded into fine powder.

Cultures Used:

Pathogens were collected from Dr. Vasantrao Pawar Medical College and Research centre, Nashik and from Culture Collections of Department of Microbiology, K.T.H.M College, Nashik. The pathogens used were as follows- *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Xanthomonas citri*, *Citrobacter divergence*, *Vibrio cholerae*, *Candida albicans* and *Aspergillus niger*.

Extract preparations:

To prepare the extract of *C.buchanani* leaves, four organic solvents were chosen (Ethyl acetate, Methanol, Acetone, Ethanol). 25gm of dried powder was taken into cotton thimble. It was extracted with 250ml of each of solvent using soxhlet apparatus¹⁰. The temperature of thermostat was maintained at minimum for the evaporation of the solvent as higher temperature will adversely affect the extracts. Rotatory vacuum evaporator was used for solvent evaporation to dryness. Extracts were dissolved in respective quantity of DMSO to make final stock concentration equal to 300µg/ml and were stored in sterile air tight container at 4°C.

Antimicrobial activity evaluation:

The antimicrobial activity of *C.buchanani* leaves extracts against the ten pathogens was evaluated by agar well diffusion technique³. The procedure was carried by following CLSI guidelines. Sterile Mueller Hinton agar plates were used. Suspensions of each organism were spread on different plates using sterile cotton swabs. The plates were then inverted for 5 min so that microorganisms adhere to surface and also to avoid moisture content. Wells were digged on plates using sterile borer (6mm) and 25µl extracts were inoculated in the wells. The whole procedure was carried under sterile condition in laminar air flow. Prediffusion of plates was done at 4°C for 30 minutes in refrigerator. After prediffusion all the plates were incubated at 37°C for 24 hours. After incubation, the inhibition zones were measured and recorded using digital vernier caliper. This activity was performed in triplicates.

Synergistic Activity Evaluation:

The synergistic effect against the different microbial pathogens was evaluated using plant extracts in combinations. The evaluation was done using agar well diffusion technique. The extracts were aseptically mixed with other in equal proportion (1:1)¹. Observations were measured and recorded and the zones of inhibition were measured using digital vernier caliper. This activity was performed in triplicates.

Potentiation Activity Evaluation:

Well diffusion technique was used for evaluating potentiation activity of extracts¹. The plant extracts were mixed with the antibiotic Gentamicin in equal proportion (1:1) aseptically. These combinations were used to examine the potentiation effect against the chosen ten pathogens. Antibiotic concentration used was 25µg/ml. Gentamicin was used as a control. Observations were measured and recorded and the zones of inhibition were measured using digital vernier caliper. This activity was performed in triplicates.

Analysis of Phytochemical Constituents:

Phytochemicals are active chemical compounds present in a plant that account for its medicinal properties. Phytochemicals were analysed by following standard procedures.

For Alkaloids: Extract was mixed with 2 ml of Wagner's reagent. Reddish brown colored precipitate indicates the presence of alkaloids¹⁷. **For Saponins:** To 2ml of extract was added 6ml of water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins¹⁶. **For Tannins:** Five ml of the extract and a few drops of 1% lead acetate were added. A yellow precipitate was formed, indicates the presence of tannins. **For Flavonoids:** To one ml of the extract, a few drops of dilute sodium hydroxide was added. An intense yellow colour was produced in the plant extract, which become colourless on addition of a few drops of dilute acid. This indicates the presence of flavonoids. **For Anthraquinones:** Five ml of the extract was hydrolysed with diluted Conc. H₂SO₄ extracted with benzene. 1 ml of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones. **For steroids:** One ml of the extract was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids. **For Triterpenes:** Ten mg of the extract was dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was added following the addition of 2 ml of Conc.H₂SO₄. Formation of reddish violet colour indicates the presence of triterpenoids¹². **For Glycosides:** 5 ml of extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayered with 1 ml of concentrated H₂SO₄. A brown ring of the interface indicates a deoxy sugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer¹⁷. **For Phytosterol:** 1ml of extract was treated with drops of chloroform, acetic anhydride and conc. H₂SO₄ and observed for the formation of dark pink or red colour¹⁶.

RESULTS AND DISCUSSION**Antimicrobial Activity:**

The result of antimicrobial activity indicated that the maximum zone of inhibition was observed in ethyl acetate extract followed by methanol, acetone, and ethanol extracts. Significant variation was seen in antimicrobial activity of plant extract depending upon solvent used. It was found that *C. buchanani*

showed broad spectrum antimicrobial activity against 9 out of 10 pathogens (Table 1). As compared to activity aqueous extract of *C. buchanani* studied by Sittiwet and Puangpronpitag¹⁵, *P. vulgaris*, *K. pneumoniae* and *S.aureus* has shown very good inhibitory effect with our organic solvents.

Table 1: Antimicrobial susceptibility testing of different *C.buchanani* leaves extracts (Mean ± Standard deviation; inhibition zone diameter in mm)

Extracts→	Ethyl acetate	Methanol	Acetone	Ethanol
Pathogens ↓				
<i>Escherichia coli</i>	20.5 ± 1.0	14.2 ± 0.5	13.6 ± 0.3	11.5 ± 0.8
<i>Staphylococcus aureus</i>	22.8 ± 1.0	18.1 ± 0.7	13.4 ± 1.3	10.2 ± 1.2
<i>Bacillus subtilis</i>	17.1 ± 0.7	14.3 ± 2.0	12.1 ± 0.4	11.5 ± 1.0
<i>Proteus vulgaris</i>	27.5 ± 1.0	21.6 ± 1.0	16.8 ± 1.5	15.3 ± 1.2
<i>Citrobacter divergence</i>	21.2 ± 0.8	15.5 ± 0.7	11.1 ± 1.0	12.1 ± 1.0
<i>Xanthomonas citri</i>	27.6 ± 0.3	20.4 ± 0.5	15.5 ± 1.0	14.2 ± 1.5
<i>Klebshiella pneumoniae</i>	25.3 ± 1.0	20.2 ± 2.0	15.1 ± 0.5	14.1 ± 1.0
<i>Vibrio cholerae</i>	26.5 ± 0.5	20.5 ± 2.0	16.3 ± 0.3	14.3 ± 2.0
<i>Candida albicans</i>	20.2 ± 0.8	13.1 ± 1.5	-	12.1 ± 1.0
<i>Aspergillus niger</i>	15.1 ± 0.6	12.3 ± 1.0	-	-

Synergistic activity:

Combinations of ethyl acetate extracts have shown high synergistic effect against all the pathogens as compared to antimicrobial activity of ethyl acetate alone. Ethyl acetate+Methanol combination had shown maximum synergistic effect against all the tested pathogens as compared to ethyl acetate alone whereas Acetone + Ethanol combination had shown antagonistic effect. Also, maximum synergistic effect was seen in Methanol+Ethyl acetate combination of extract against *Proteus Vulgaris*. Hence, the synergistic effect of *C.buchanani* leaves extracts when combined was additive against the pathogens as compared to antimicrobial activity of extract alone (Table 2, Figure 1,2,3 & 4).

Table 2: Synergistic effect of *C.buchanani* extracts on different pathogens (Mean ± Standard deviation; inhibition zone diameter in mm)

Combinations→	Ethyl Acetate + Methanol	Ethyl Acetate + Acetone	Ethyl Acetate + Ethanol	Methanol + Acetone	Methanol + Ethanol	Ethanol + Acetone
Pathogens ↓						
<i>Escherichia coli</i>	17.8 ± 0.8	16.5 ± 1.2	14.1 ± 0.9	12.5 ± 0.8	12.1 ± 0.8	11.1 ± 0.4
<i>Staphylococcus aureus</i>	22.1 ± 1.0	18.6 ± 0.5	16.5 ± 1.0	14.2 ± 1.3	15.3 ± 1.2	11.5 ± 1.0
<i>Bacillus subtilis</i>	20.5 ± 1.0	17.3 ± 1.1	15.6 ± 0.9	12.3 ± 0.9	13.5 ± 1.0	10.4 ± 0.8
<i>Proteus vulgaris</i>	25.2 ± 1.2	19.3 ± 1.0	18.4 ± 0.5	14.1 ± 0.7	15.6 ± 0.9	13.5 ± 1.0
<i>Citrobacter divergence</i>	22.6 ± 1.1	20.5 ± 1.9	14.2 ± 1.2	13.4 ± 1.5	13.3 ± 1.1	11.2 ± 0.8
<i>Xanthomonas citri</i>	23.3 ± 0.9	19.2 ± 0.9	17.2 ± 1.0	15.5 ± 1.1	14.1 ± 1.0	10.1 ± 0.9
<i>Klebshiella pneumoniae</i>	20.2 ± 0.9	19.1 ± 0.6	15.4 ± 1.3	12.2 ± 1.4	13.5 ± 1.3	10.1 ± 1.2
<i>Vibrio cholerae</i>	21.3 ± 0.8	17.4 ± 0.7	12.1 ± 1.1	11.2 ± 1.2	12.4 ± 0.6	11.1 ± 0.9
<i>Candida albicans</i>	21.1 ± 0.5	16.3 ± 1.2	-	-	-	-
<i>Aspergillus niger</i>	15.3 ± 0.9	12.1 ± 1.5	-	-	-	-

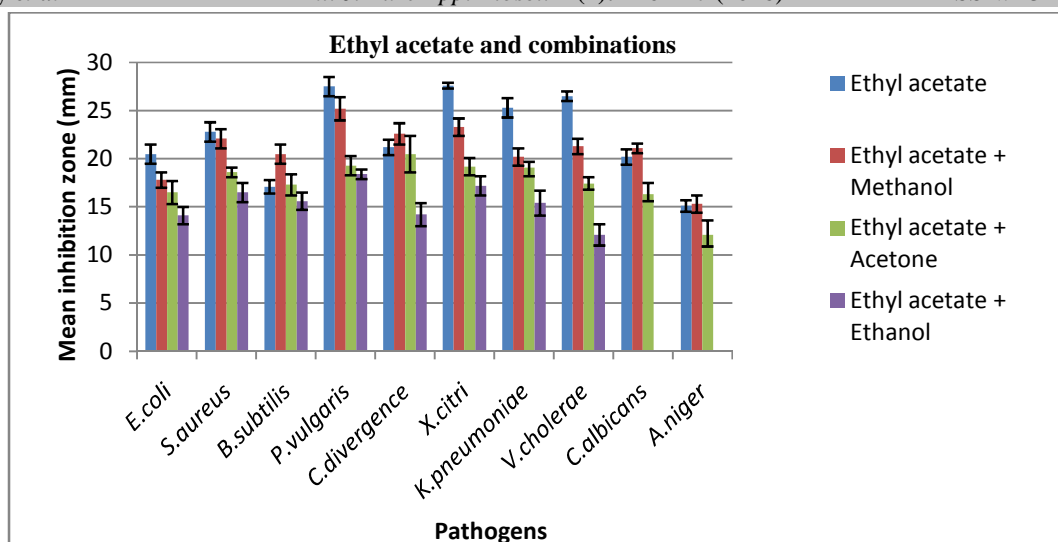


Fig 1: Synergistic activity of Ethyl acetate combinations

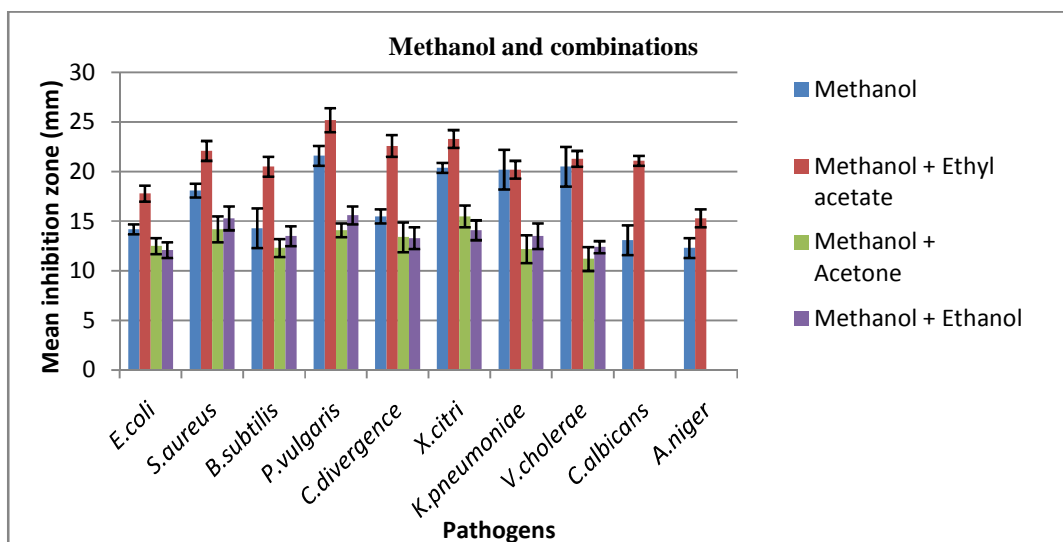


Fig 2: Synergistic activity of Methanol combinations

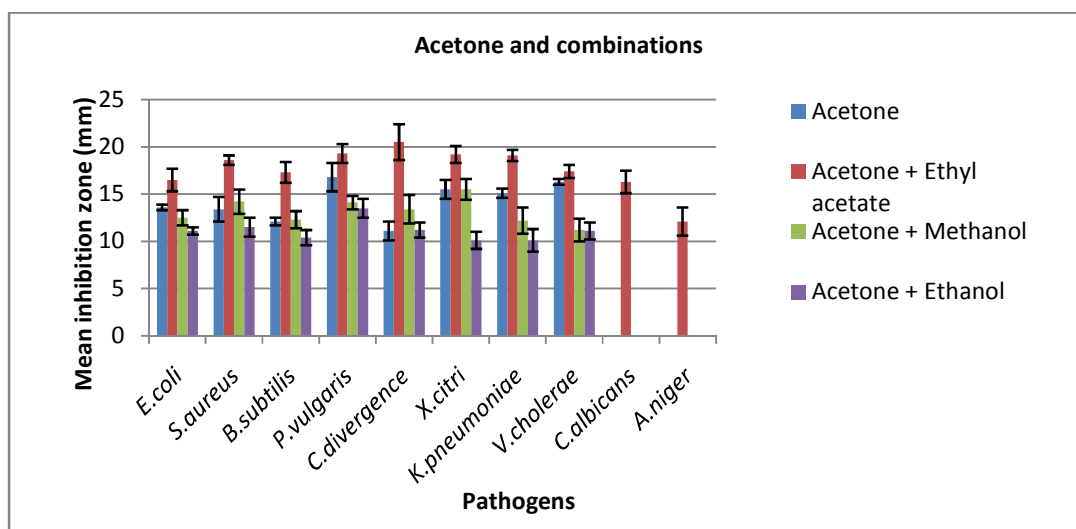


Fig 3: Synergistic activity of Acetone combinations

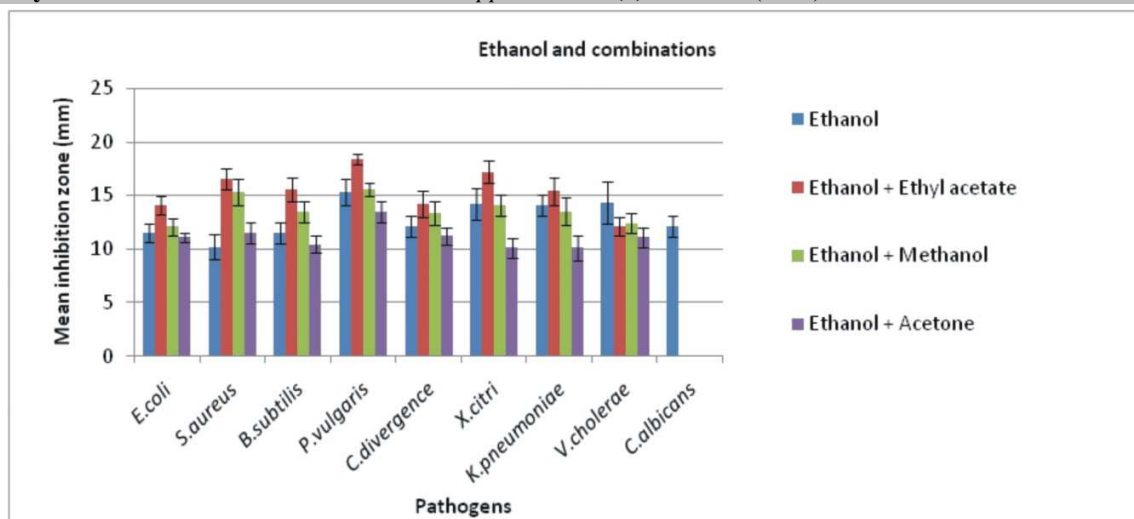


Fig 4: Synergistic activity of Ethanol combinations

Potential activity:

The observation of potential activity showed that the combination of extracts with Gentamicin could not potentiate its activity against the chosen pathogens. Only ethyl acetate combination was able to potentiate the effect of Gentamicin against single pathogen, *Proteus vulgaris* (Table 4).

Table 3: Potentiation effect of *C.buchanani* leaves extracts on a standard antibiotic, Gentamicin (Mean \pm Standard deviation; inhibition zone diameter in mm)

Extract \rightarrow Pathogens \downarrow	Ethyl acetate + Gentamicin	Methanol + Gentamicin	Acetone + Gentamicin	Ethanol + Gentamicin	Gentamicin alone
<i>Escherichia coli</i>	22.2 \pm 1.4	20.7 \pm 1.0	18.4 \pm 0.5	15.2 \pm 0.7	32.3 \pm 0
<i>Staphylococcus aureus</i>	24.8 \pm 0.5	22.1 \pm 1.2	16.4 \pm 1.0	15.1 \pm 0.5	27.1 \pm 0
<i>Bacillus subtilis</i>	17.1 \pm 0.8	19.9 \pm 0.7	15.1 \pm 0.5	14.8 \pm 0.7	32.1 \pm 0
<i>Klebsiella pneumoniae</i>	23.3 \pm 1.4	22.4 \pm 0.5	20.6 \pm 1.0	16.5 \pm 0.5	29.4 \pm 1.4
<i>Citrobacter divergence</i>	21.5 \pm 0.5	19.8 \pm 0.7	18.4 \pm 0.5	17.2 \pm 1.0	26.5 \pm 0.7
<i>Xanthomonas citri</i>	24.4 \pm 0.4	20.3 \pm 0.7	20.2 \pm 0.5	18.8 \pm 0.8	32.1 \pm 0
<i>Proteus vulgaris</i>	29.7 \pm 0.3	22.1 \pm 0.7	20.8 \pm 1.0	19.1 \pm 0.5	32.5 \pm 0
<i>Vibrio cholerae</i>	22.9 \pm 0.7	20.5 \pm 0.5	16.1 \pm 0.4	18.6 \pm 0.5	27.5 \pm 0.7
<i>Aspergillus niger</i>	20.6 \pm 0.4	18.2 \pm 0.8	-	-	20.1 \pm 0
<i>Candida albicans</i>	15.3 \pm 0.5	12.4 \pm 0.5	-	-	-

Phytochemical analysis:

Alkaloids and saponins were present in all the extracts whereas other constituents such as tannins, triterpenes, flavonoids, anthraquinones, phytosterols, glycosides and steroids have varied in their presence. This analysis indicated that the phytochemical constituents of extracts have shown significant variation. Thus, *C.buchanani* is highly rich in its phytochemical contents and thereby has variety of medicinal applications (Table 4). Our phytochemicals results of methanol extract are also in accordance with results of Vinayaka et al, which also had alkaloids and saponins present in their extract.

Table 4: Phytochemical analysis of *C.buchanani* leaves extracts (Present = '+' and Absent = '-')

Extracts→	Ethyl acetate	Methanol	Acetone	Ethanol
Phytochemicals ↓				
Alkaloids	+	+	+	+
Saponins	+	+	+	+
Tannins	-	+	+	-
Triterpenes	+	-	-	+
Flavonoids	-	+	+	-
Anthraquiones	+	+	-	-
Phytosterols	+	+	+	-
Glycosides	+	+	-	+
Steroids	-	-	+	-

CONCLUSIONS

Among all the pathogens tested against all the organic extracts prepared from leaves of *Cryptolepis buchanani*, *Proteus vulgaris* was found to be most sensitive with maximum zone of inhibition and *Aspergillus niger* was resistant to them. This result indicates that the antimicrobial activity varied depending upon the type of solvent used. Ethyl acetate extracts exhibited maximum antimicrobial activity than the other solvent extracts of *Cryptolepis buchanani*, so we can conclude that ethyl acetate extract was more suitable solvent for maximum extraction of active metabolites which are responsible for antimicrobial activity. From the results obtained it was concluded that the combinations of ethyl acetate with every extract had shown highest synergistic effect against *Xanthomonas citri*, *Bacillus subtilis* and *Proteus vulgaris*. Antagonistic effect was observed against the fungal pathogens used for synergistic combinations. In conclusion the synergistic effect of leaves extracts combinations of *C. buchanani* against pathogens was observed to be additive against the pathogens as compared to antimicrobial activity of extracts alone. All the extracts showed the presence of alkaloids and saponins. Thus the results indicate that *C. buchanani* is highly rich in the diversity of phytochemical constituents. These compounds may elicit a long range of different effects on man and may contribute to more medicinal uses.

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