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Research Article

Antimicrobial Assessment and Phytochemical Screening of Medicinal Plants and *Ganoderma lucidum*

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Abstract

Nepal has always relied on traditional medicinal plants, herbs, and mushrooms that are locally available to treat numerous diseases. This study thus aimed to explore the antimicrobial properties of the plants and fungi that are commonly used as traditional medicines. Antimicrobial properties of 5 plants (*Curcuma caesia*. *Acorus calamus*, *Moringa oleifera*, *Terminalia chebula*, and *Tinospora cordifolia*) and 1 fungus (*Ganoderma lucidum*) were screened against 4 ATCC bacterial culture (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*). The extracts were prepared using Soxhlet's apparatus in two solvents, i.e. ethanol and distilled water. Out of which, distilled water of *Acorus calamus* had the highest percentage yield. All extracts showed antimicrobial properties against *S. aureus*. The highest potential was observed in the ethanolic extract of *Curcuma caesia* against *S. aureus* with the zone of inhibition of 22mm. The ethanolic extract of *Tinospora cordiofolia* showed promising antimicrobial against gram-negative bacteria*: E.coli*, *P. aeruginosa* and *K. pneumoniae* with the zone of inhibition 15mm, 15mm and 14mm respectively. The distilled water extract of *Acorus calamus* showed the highest antimicrobial activity against *P. aeruginosa* with a zone of inhibition of 18mm. In the case of phytochemical screening, both the extracts of *Tinospora cordiofolia* showed the highest phytochemicals (Terpenoids, Saponins, Coumarin, Flavonoids, Alkaloids, and Tannins). The experiment confirmed the efficacy of selected plants and fungus extract as a natural antimicrobial against all the test organisms used, suggesting the possibility of employing them in novel drug development against the treatment of infectious diseases caused by the test organisms.

Keywords: antimicrobial; zone of inhibition; phytochemicals; soxhlet's extraction

Introduction

Nepal is home to 7000 species of flowering plants and 4000 species of non-flowering plants, among which roughly about 1500-1800 species are being used by the local residing communities to treat various illnesses. Moreover, 100 plants/plants parts and more are annually traded in and from Nepal as Medicinal and Aromatic Plants (Pyakurel *et al.*, 2018). In Nepal, more than 80% population uses traditional medicines mostly from medicinal plants, as they are the primary source of health care for many people. Medicinal plants are also an integral part of culture in the country, and most people do use plants as an alternative to western medicine (Khadka *et al*., 2021).

Medicinal plants and herbs are the type of plants that consist of antimicrobial substance in them. An antimicrobial substance is an agent that kills microorganisms or stops their growth. (Purssell 2020). There are also instances where medicinal plants can be anti-inflammatory, antioxidant and antiviral (Mandeville *et.al*., 2018) (Mujeeb *et al*., 2014) (Bag *et.al*., 2013). Plants are autotrophic organisms, and have two sets of metabolism: one is primary

metabolism that is present in all living beings and secondary metabolism that enable plants to produce and accumulate diverse chemical compounds (Mendoza and Silva, 2018). Primary metabolites are those metabolites that are necessary for normal functioning of plants, some examples of it are amino acids, nucleotides, sugars and lipids. Similarly, the secondary metabolites refers to the organic molecules that don't seem to have a direct function to growth and development of plant, but are suspected to help plant protect from diseases. (Adetunji et al., 2020). Some of active secondary metabolites are alkaloids, flavonoids, tannins, terpenes and quinones that have shown promising results against resistance developing microorganisms like *Escherichia coli*, *Staphylococcus aureus*, *Psuedomonas aeruginosa*, and *Salmonella* (Compean and Ynalvez, 2014).

Nepal is suffering from high burden of antimicrobial resistance due to inappropriate use of antibiotics, and inaccessibility to proper health care facilities (Rijal *et al*., 2021). According to the CDC, more than two millions people suffer illness due to antibiotic resistance pathogens in the United States, and the death toll associated with antibiotic resistance is 23,000 every year (Dadgostar 2019). The WHO speculated that there is possibility of 10 millions death per year by the year 2050 due to antibiotic resistance pathogens. However, in Nepal, such concrete surveillance has not been done yet but there seems to be evidences that highlights the raising burden of antimicrobial resistance pathogens in the country (Acharya and Wilson, 2019). In a country that relies heavily on traditional medicines and medicinal plants, the importance of rationalizing these claims are pivotal. Furthermore, the COVID-19 pandemic have somewhat increased the use of medicinal plants in Nepal, and made more people aware about their effectiveness. In a recent survey conducted by Khadka and his co-workers also reported that in and after the pandemic, there has been a significant increase on the use and knowledge of medicinal plants among peoples here at Nepal (Khadka *et al*., 2021). This further paves way to the researches and scientists to now rationalize the claim of the medicinal plants being effective and also look onto the other end of spectrum consisting of its side effects as well.

Additionally, taking the step to a good direction, Nepal formulated the National Action Plan for the Containment of Antimicrobial Resistance in Nepal in 2016, and set out various objectives. Among which, one objective emphasizes on researches on possible new alternatives to allopathic medicines. Thus, our study also emphasizes on rationalizing the antimicrobial activities of locally available *Curcuma caesia* (Black turmeric), *Ganoderma lucidum* (Red mushroom), *Acorus calamus* (Bojo), *Moringa oleifera* (Sitalchini), *Tinospora cordifolia* (Gurjo), and *Terminalia chebula* (Harro) against four emerging drug resistance bacteria as namely *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Murray *et al*., 2019).

Materials and Methods

The research was conducted from March 22nd to June 25th at Department of Microbiology, St. Xavier's College, Maitighar, Kathmandu, Nepal. The test organisms used in this study were *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC BAA 1705.88.18), and *Pseudomonas aeruginosa* (ATCC 27853) which were received from National Public Health Laboratory, Teku, Kathmandu, Nepal.

Sample Collection and Processing

Common medicinal plants that are frequently used as traditional medicine were collected as samples. The samples used were *Curcuma caesia*, *Ganoderma lucidum*, *Acorus calamus*, *Moringa oleifera*, *Tinospora cordifolia*, and *Terminalia chebula*. Among six samples, *Ganoderma lucidum* was brought from Nepalgunj while rest of five samples were collected from different areas of Kathmandu valley. The plants were identified by Mr. Brajesh Shrestha, Head of Department (Department of Biology) at St. Xavier's College with the help of reference from Dictionary of Nepalese Plant Names by Mr. Keshab Shrestha. The sampling technique used was purposive sampling, where samples were collected considering the purpose of study. The collected samples were then washed with distilled water and disinfected by 70% ethanol. Washed samples were then oven dried at 50°C for 3 days.

Sample Extract Preparation

Dried plants were grinded using grinder into fine powder, 25 grams of plant powder was kept in the thimble made up of blotting paper and inserted into the extraction chamber of the Soxhlet's apparatus. Following this, the solvents (distilled water and 80% ethanol) were added in the Soxhlet's round bottom flask respectively, which is then attached to Soxhlet extraction chamber and condenser on an isomantle, where the heat is applied and Soxhlet is left to run for about 5 cycles. (Redfern *et al*.,2014). The percentage yield of the extract was calculated by the given formula :

$$\%\ \text{Yield of Plant Extract} = \frac{Dry\ weight\ of\ extract}{Dry\ weight\ of\ plant} \times 100$$

Preparation of Extracts of Varying Concentration

One gram of each plant extract was taken and was dissolved in 1ml of Dimethyl Sulphoxide (DMSO) for the preparation of working solution. Thus 1000 mg/ml of stock was obtained as a standard concentration of extracts. For different concentrations i.e., 200 mg/ml, 100 mg/ml, and 50mg/ml the amount of extract from 1000mg/ml and DMSO was calculated using formula; S1V1=S2V2

Where, S1= concentration of standard solution

V1= volume of standard solution required to make solution of required concentration

S2= concentration of required solution

V2= final volume of required solution to be a made.

Phytochemical Screening of The Prepared Extracts (Compean and Ynalvez, 2014; Yadav et al., 2014)

i. *Detection of Flavonoid*: To 1ml plant extract, few drops of dilute HCl, followed by the addition of dilute acid. Appearance of yellow color indicated the presence of flavonoid.

ii. *Detection of Tannins*: Extract of 5-10ml was boiled in a test tube and filtrate. To which, few drops of 5% ferric chloride solution was added. Formation of brownish green or blue-black coloration indicated the presence of tannin.

iii. *Detection of Glycosides*: To 2 ml of glacial acetic acid, one drop of 2% Ferric chloride solution was mixed and 5 ml of aqueous extract. Then 1 ml of concentrated sulphuric acid. Presence of brown ring at the interface of the layers indicated the presence of glycosides.

iv. *Detection of Terpenoids by Salkowski's Test*: To 2 ml extract, 2 ml of chloroform was added. To this, 2ml of sulphuric acid was added to the layer. A reddish-brown coloration at the interface indicated the presence of terpenoids.

v. *Detection of Saponins by Foam test:* To 5 ml extract, 5ml of distilled water was mixed and heated in a test tube and shaken vigorously. The formation of stable foam indicated the presence of saponin.

vi. *Detection of Alkaloids using Marquis reagent:* To prepare Marquis reagent, 0.5 ml of formaldehyde was added to 5ml of concentrated sulphuric acid. To this reagent, 5 ml of extract was added, and appearance of green color indicated the presence of alkaloids.

vii. *Detection of Anthraquinone:* To extract that was dissolved in DMSO. 0.5 ml of ammonia solution was added to the mixture. Then the mixture was shaken vigorously. Formation of reddish color indicated the presence of anthraquinone.

viii. *Detection of Coumarin:* 1ml plant extract was mixed with 1ml of 10 % NaOH solution. Formation of yellow coloration indicated the presence of coumarin.

ix. *Detection of Starch:* 3-4 drops of iodine solution were added to 0.5ml of stock solution of plant extract. Formation of blue color indicated the presence of starch.

x. *Detection of Quinones:* 1ml plant extract was added to 1 ml of concentrated sulphuric acid and formation of red color indicated the presence of quinones.

Identification and Preparation of Microbial Culture

The test bacteria were inoculated in nutrient agar and was incubated at 37°C for 24 hours and was gram stained and were subjected to various biochemical tests in order to identify it. The test organisms were maintained at 4°C. Active cultures for each bacterial species were prepared by transferring a loop full of colony from the stock cultures to test tubes of nutrient broth and incubated at 37°C overnight & test tubes were then compared with 0.5 McFarland standard.

Determination of Antibacterial Activity

With the help of sterile swab, MHA agar plates were inoculated with isolated test organism and was left for 10 minutes to absorb. Using sterile well borer 4 wells were made on agar plates. 50 µl of extract of different concentration (200mg/ml, 100mg/ml and 50mg/mml)

and DMSO as negative control were loaded in each different well and left at room temperature for about 1 hour for absorption of extract. The plates were incubated at 37°C for 24 hours and zone of inhibition were measured and recorded.

Result and Discussion

Percentage Yield of Extracts

The samples were extracted in two solvents namely 80% ethanol and distilled water, percentage yield of all the extracts were calculated (Table 1). The highest percentage yield of 1.7 % was obtained from the ethanolic extract *Terminalia chebula*. Likewise, the least percentage yield of 0.36% was obtained from the ethanolic extract of *Ganoderma lucidum*. The overall low yield extraction percentage can be attributed to the low weight of samples that were used in the study, also the affinity between solvent polarity and the compounds used plays an important role in extraction process (Złotek *et al*., 2016).

Soxhlet apparatus was used for the extraction process because large amount of extracts can be obtained with smaller amount of solvent and applicable to plant materials that are heat stable. The solvent used depends upon the nature of compounds to be isolated. Distilled water was used as solvent as it dissolves a wide range of substances; hence is cheap, nontoxic, nonflammable, and highly polar. Ethanol is also considered as the best solvent for extraction as per the studies. It can preserve itself at a concentration above 20% and hence is nontoxic at low concentration, and as small amount of heat is required for concentrating the

extract.(Abubakar and Haque, 2020). As both being polar solvents somewhat similar yield extract was obtained.

Table 1: Percentage yield of extracts

Solvent used	Method of extraction	*Acorus calamus*	*Tinospora cordifolia*	*Terminalia chebula*	*Ganoderma lucidum*	*Moringa oleifera*	*Curcuma caesia*
Ethanol	Soxhlet's	0.76	1.7	0.90	0.36	0.68	0.85
Distilled water	Soxhlet's	1.59	1.50	0.36	0.46	0.75	0.85

Table 2: Phytochemical screening of ethanolic and distilled water extracts

	Botanical name	Solvent used	Phytochemical tests									
			P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
1.	*C. caesia*	Ethanol	+	+	-	+	+	+	-	-	-	-
		Distilled water	+	-	+	+	-	+	-	-	-	-
2.	*A. calamus*	Ethanol	-	+	+	-	-	-	-	-	-	-
		Distilled water	-	+	+	-	-	+	-	-	-	-
3.	*M. oleifera*	Ethanol	+	+	-	-	+	+	-	+	-	-
		Distilled water	-	+	-	+	-	+	-	+	-	-
4.	*T. cordifolia*	Ethanol	-	-	+	-	-	-	-	+	-	-
		Distilled water	+	+	+	-	+	+	-	+	-	-
5.	*T. chebula*	Ethanol	+	-	-	-	-	-	-	+	-	-
		Distilled water	+	-	-	-	-	-	-	-	-	-
6.	*G. lucidum*	Ethanol	+	-	-	+	+	-	-	-	-	-
		Distilled water	+	+	-	+	+	-	-	-	-	-

P1 = Flavonoids; P2 = Tannins; P3 = Glycosides; P4 = Terpenoids; P5 = Saponins; P6 = Alkaloids; P7 = Anthraquinone; P8 = Coumarin; P9 = Starch; P10 = Quinones.

Phytochemical Screening of Ethanolic and Distilled Water Extracts

The phytochemical screening of chemical constituents of used samples extracts were studied quantitatively. All the plants revealed the presence of active compounds. The ethanolic extract of *C. caesia* indicated the presence of flavonoids, tannins, alkaloids, glycosides, and terpenoids which is the highest amount of phytochemicals present among all the extracts. Similarly, the distilled water extracts of *T. cordiofolia* showed the highest amount of phytochemicals, among phytochemicals that were screen, 6 were present in this extract (Table 2).

Consequently, the phytochemical screening of different extracts in different solvents varied. In the extract of *C. caesia*, phytochemicals such as flavonoids, tannins, glycosides and terpenoids were present in both ethanolic and distilled water and alkaloids was present only in distilled water. This could be because of higher polarity of water that allowed the polar alkaloids to get dissolve (Wakeel *et al*., 2019). Saponins, anthraquinone, coumarin, starch and quinones were absent in both.

In case of *A. calamus*, tannins, glycosides and alkaloids were present in both ethanol and distilled water. Saponins was present only on ethanolic extract. This could be because some saponins with non-polar moieties have reduced polarity hence thus get dissolved in less polar solvent like ethanol rather than on high polar solvent water (Ashour *et al*., 2019). Flavonoid, terpenoid, coumarin, starch and

quinones were completely absent in both ethanolic and distilled water. The reason for which can be errors caused during the time of extraction. These findings were in agreement with previous studies of done by (Balaji and Shanthakumari, 2021). In the extract of *M. oleifera*, phytochemicals such as tannins, alkaloids and coumarin were present in both extract and flavonoids was present only in ethanol. These findings also resemble findings by Shanriar *et al*. (2012). Similarly, tannins, terpenoids, saponins and alkaloids were present in both extracts of *T. cordifolia*. Whereas, glycosides, anthraquinone, coumarin, starch and quinones were completely absent, the absence of these mentioned phytochemicals can be reasoned for their non-polar moieties that did not dissolve in the polar solvents used in our study. Flavonoids and coumarin were only present in ethanolic extract. These findings were similar to the study of Yadav and Agarwala (2011). In the fruit extract of *T. chebula*, flavonoids and tannins were present in both ethanol and distilled water extracts whereas glycosides, alkaloids and coumarin was only present in ethanolic extract. Tannins was only present in distilled water, reason for which is mainly due to tannins being polar which mix well with polar solvents like water (Feng *et al*., 2019).

Antimicrobial activity of samples extracts

The samples were investigated to evaluate their antimicrobial activity against pathogenic bacteria including one strain of Gram-positive bacteria (*Staphylococcus aureus*) and three strains of Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) using disc diffusion method. Evaluation of antimicrobial activity of these plants extracts was recorded in the Table 3 and 4.

T**able 3:** Antimicrobial activity of ethanolic extracts

S.N.	Botanical name	Concentrations (mg/ml)	Zone of inhibition (in mm)			
			S. aureus	*E. coli*	*P. aeruginosa*	*K. pneumoniae*
1.	*C. caesia*	200	22	-	13	11
		100	18	-	12	9
		50	14	-	10	-
		DMSO	-	-	-	-
2.	*A. calamus*	200	12	11	13	15
		100	12	-	12	13
		50	11	-	-	-
		DMSO	-	-	-	-
3.	*M. oleifera*	200	12	-	13	15
		100	11	-	-	14
		50	10	-	-	13
		DMSO	-	-	-	-
4.	*T. cordifolia*	200	15	15	15	14
		100	10	14	14	14
		50	9	-	13	-
		DMSO	-	-	-	-
5.	*T. chebula*	200	16	12	13	13
		100	14	10	11	-
		50	13	-	-	-
		DMSO	-	-	-	-
6.	*G. lucidum*	200	14	13	14	14
		100	10	11	10	12
		50	-	-	-	-
		DMSO	-	-	-	-

Table 4: Antimicrobial activity of distilled water extracts

S.N.	Botanical name	Concentrations (mg/ml)	Zone of inhibition (in mm)			
			S. aureus	*E. coli*	*P. aeruginosa*	*K, pneumoniae*
1.	*C. caesia*	200	13	16	10	14
		100	11	13	10	12
		50	10	10	-	10
		DMSO	-	-	-	-
2.	*A. calamus*	200	16	14	18	13
		100	13	-	-	12
		50	11	-	-	-
		DMSO	-	-	-	-
3.	*M. oleifera*	200	20	-	11	14
		100	18	-	-	12
		50	12	-	-	11
		DMSO	-	-	-	-
4.	*T. cordifolia*	200	15	-	13	-
		100	13	-	11	-
		50	10	-	-	-
		DMSO	-	-	-	-
5.	*T. chebula*	200	12	10	10	-
		100	11	-	-	-
		50	10	-	-	-
		DMSO	-	-	-	-
6.	*G. lucidum*	200	12	10	11	10
		100	11	-	-	-
		50	-	-	-	-
		DMSO	-	-	-	-

Ethanolic and aqueous extracts were preferred as an extraction solvent because of its high polarity and redial availability. Ethanol also contains both polar and non-polar groups, which makes it efficient choice of solvent. Distilled water is however highly polar thus more polar phytochemicals compounds can be extracted on it (Nawaz *et al*., 2020). Soxhlet's extraction using distilled water as solvent have been reported to generate higher extraction efficiency (Borges *et al*., 2020). In our study, all the prepared extracts showed antimicrobial activity against gram positive *S. aureus***.** Except, *C.caesia* and *M.oleifera* ineffectiveness against test organism *E.coli*, all ethanolic extracts showed antimicrobial activity against all the test gram negative organisms. The resistance shown by *E.coli* could be attributed to its cell wall structure as it has an effective permeability barrier, comprised of a thin lipopolysaccharide exterior membrane, which could restrict the penetration of the plant extract. A similar study showed that the zone of inhibition of ethanol *C. caesia* extract on *E.coli* at 100mg/ml produced zone of inhibition 12mm which was least effective (Kaur *et al*., 2018). Despite being ineffective against *E.coli*, the ethanolic extract of this plant showed the maximum zone of inhibition of 22mm at concentration 200mg/ml against S. aureus. The ethanolic and distilled water extracts of *Acorus calamus* showed the zone of inhibition ranging from 11-15 mm at concentration 50-200 mg/ml concentration against all the test organism used. However, the zone of inhibition was limited to concentration 100 mg/ml and 200 mg/ml against the three gram negative organism used in both the solvents used. The plant extract of *A. calamus* however, showed zone of inhibition against *E. coli* at only the highest concentration

of 200mg/ml with 11mm. The limited activity against *E.coli* was also reported by Muchtaromah *et al*. (2019). The study by Maharjan *et al*. (2013) showed no zone of inhibition against *K, pneumoniae* and *P. aeruginosa*, however our study showed zone of inhibition against it at higher concentration.

Similar to other extract, the ethanolic and distilled water *Ganoderma lucidum* extracts antimicrobial activity against all the test organisms used. However, the antimicrobial activity was mostly limited to higher concentration of 100 mg/ml and 200 mg/ml. The extract also showed promising activities against gram negative organisms like *E.coli, P. aeruginosa* and *K. pneumoniae* with zone of inhibition ranging from 10mm-14mm. Similar results against *S. aureus* and *P. aeruginosa* were reported by Djide *et al*. (2014) with ethanol extract of *G. lucidum*. In the distilled water extract, similar antimicrobial activity was observed. But the antimicrobial effect was mostly limited to higher concentration of 200mg/ml. Moreover, it showed zone of inhibition against gram positive, *S. aureus* in two different concentration of 100 mg.ml and 200 mg/ml, thus supporting the result observed by Kamble *et al*. (2011) where they found distilled water extract of *G. lucidum* to be effective against *S. aureus* with 18.5 mm zone of inhibition. Our study also confirmed the *G. lucidum* extract having the potential to exhibit antimicrobial activity against gram negative organism like *E. coli* and *K. pneumoniae*.

Both the ethanolic extract of *T. cordifolia* showed antimicrobial activity against all the test organisms. However, the concentration at which it exhibited antimicrobial activity did varied. In the case of *S. aureus*, the ethanolic extract was effective from concentration 50mg/ml with 9mm, and increasing to 10mm and 15mm, as the concentration went up to 100mg/ml and 200mg/ml, respectively. In the case, of *P. aeruginosa* as well, the ethanolic extract showed zone of inhibition at all concentrations used, the zone of inhibition was 13mm, 14mm and 15mm with increasing concentrations. And the extract was effective against *E. coli* and *K. pneumoniae* at only two concentrations of 100mg/ml and 200mg/ml. The antimicrobial activity against gram negative organism by *T. cordiofolia* is also reported by Gunda and Kommidi (2020). The antimicrobial activity was comparatively low in distilled water extract, and showed no zone of inhibition against *E. coli* and *Klebsiella*. Prajwala *et al*. (2018) also inferred similar result of distilled water extract showing no antimicrobial property against *E. coli*. The extract was most effective on gram positive, *S. aureus* at all concentrations used. However, only highest concentration (200mg/ml) was able to exhibited zone of inhibition against test gram negative organisms. The study carried out by Mishra *et al.* (2014) showed contradictory result where no zone of inhibition was shown against *E. coli, S. aureus* and *P. aeruginosa*. Our study finding reported a higher zone of inhibition than Shrestha and Lamichhane (2021) reported in the extract of *Tinospora cordifolia.*

Moringa Oleifera showed the highest zone of inhibition against *Kleibsell*a in ethanol as solvent whereas the plant showed the inhibtion of 20mm against *S. aureus* in the distilled water. As per the previous study, the maximum zone of inhibition showed by the extract against was 9mm against *S. aureus* and was 4mm against *E. coli* (Ojiako, 2014). Both the ethanolic and distilled water extract showed no effect against *E. coli*. Distilled water extract of *Terminalia chebula* showed very less activity against all the test organisms where no activity was seen against *E coli*. The zone of inhibition was showed by the plants against all the organisms in ethanolic extracts.

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Authors' Contribution

All authors contributed equally in all stages of research work, manuscript preparation, critically revision and finalization of the manuscript. Final form of manuscript was approved by all authors.

Conflict of Interest

The authors declare that they have no conflict of interest with the present study.

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