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Phytochemical Screening and Evaluation of Antioxidant and Cytotoxic Activities of *Ganoderma lucidum*

Anil Bhusal, Santosh Dhungana, Sujan Sharma, Suraj Dahal, Krishna Neupane, Sajan Maharjan

Department of Pharmacy, CIST College (Affiliated to Pokhara University), New Baneshwor, Kathmandu, Nepal

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ABSTRACT

This study aims to conduct a comprehensive investigation of the phytochemical composition of Ganoderma lucidum and evaluate its potential, antioxidant, and cytotoxic properties found in the higher altitude region of Nepal. Extraction was perform by maceration, Soxhlet extraction and maceration followed by Soxhlet using ethanol as solvent. The phytochemical screening was performed by standard method. Phytochemical screening of extract reveals that extract contains proteins, carbohydrates, terpenoids, saponins, glycosides, alkaloids. Antioxidant test was performed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay method and cytotoxicity test was done by using Brine shrimp lethality assay. Among three methods of extraction, maceration process observed to have least extractive value and Soxhlet and Soxhlet followed by macerationseems increasing extractive value. Result shows that The IC50 of DPPH scavenging assay was determined as 53.88±0.62 and for cytotoxicity LD50 value was155.89 ±1.75.This research reveals that ganoderma show pronounced antioxidant and cytotoxic activity.The findings could explain why G. lucidum extracts are used in folk medicine.

Keywords ; Phytochemicals, Cytotoxicity , Antioxidants ,Brine Shrimp

1. INTRODUCTION

According to the World Health Organization, an estimated 80% of people worldwide are interested in traditional medicine. At present, research evaluating the chemical compositions and therapeutic utilities of herbal medicines is being conducted worldwide(1)*Ganoderma lucidum*oriental fungus has a long history of use for promoting health and longevity in China, Japan, and other Asian countries(2). It is a large, dark mushroom with a glossy exterior and a woody texture. *Ganoderma lucidum*, an oriental fungus has a long history of use for promoting health and longevity in China, Japan, and other Asian countries(3).*Ganoderma lucidum* belongs to family Ganodermataceae which is a family of fungi in the order Polyporales, native to tropical and temperate regions. Basidiocarps of this genus have a laccate (shiny) surface that is associated with the presence of thick walled pilocystidia embedded in an extracellular melanin matrix. It is a large, dark mushroom with a glossy exterior and a woody texture .The fruit bodies of ganoderma species vary in shape and color (red, black, blue/green, white, yellow, and purple), host specificity, and geographic origin(4).

More than 400 different chemical compounds, such as triterpenes, polysaccharides, nucleotides, alkaloids, steroids, amino acids, fatty acids, and phenols, can be found in Ganoderma(5) These exhibit different therapeutic qualities (6). Ganoderma lucidum showed promising effect in cancer treatment , triterpene fraction depicted to have special Cytotoxic property was accounted to restrain development and metastatic prospective of breast cancer cells (MDA-231) constructing it as a potential cytotoxic agent(7). Triterpenes extracted from G. lucidum have anti-oxidative properties in vitro and can reduce oxidative damage by directly scavenging free radicals generated in the cell (8) .Triterpenoids and polysaccharides have been extensively studied because of their pharmacological effects(9)and they have been shown to possess bioactivities such as anti-tumor(10) immunoglubator(11), hepatoprotective(12), antioxidant (13), osteoclastogenesis(14), antiviral (15), and anti-inflammatory(16).The aim of this study is to evaluate the different secondary phytochemical constitute of ganoderma lucidum and evaluate the antioxidant and cytotoxicity activites . This research aims to investigate the potential of G. lucidum as a natural remedy for mitigating oxidative stress and its cytotoxic impact on cancer cells.This study could provide significant insights into its therapeutic characteristics and aid in the advancement of natural treatments for illness prevention and cancer therapy.

2. Materials and methods

2.1. Plant Sample Collection and identification

Ganoderma lucidum were collected from Malikabota Municipality, Jumla, Karnali province at an altitude of 2400m above sea level.Herbarium of the collected Species was recognized by Government of Nepal Ministry of Forests and Environment Department of Plant Resources National Herbarium and Plant Laboratories (KATH) (Species Ref. no. 461) to confirm that right species for study was collected.

2.2. Extraction method

The extraction was performed by separate processes such as Maceration extraction, Soxhlet extraction and maceration followed by Soxhlet at room temperature. Where ethanol was used as solvent(17).

2.3. Phytochemical screening

2.3.1. Test for Glycosides

To the extract, few drops of 10% NaOH were added to make it alkaline. Then, freshly prepared sodium nitroprusside was added to the solution. Presence of blue colouration indicated the presence of glycosides in the extract(18).

2.3.2. Test for terpenoids

0.2 g of each sample was mixed with 2 mL chloroform, 3 mL conc. H2 SO4. Reddish-brown coloration indicated the presence of terpenoids(19).

2.3.3. Test for alkaloids:

To a few ml of extract, 1 or 2 ml of Dragendorff s reagent was added. A prominent yellow precipitate indicated the test as positive.

2.3.4 Test for Carbohydrates

To 2-3 ml of ethanolic extract , few drops of α-napthol solution was added in alcohol, shaken, concentrated H2SO4 from sides of the test tube was added and observed for violet ring at the junction of two liquids(20).

2.3.5. Test for flavonoids

Five millilitres (5 mL) of the aqueous solution of the water extract was mixed with 0.1 g of metallic zinc and 8ml of concentrated sulphuric acid. The mixture was observed for red colour as indicative of flavonols(21).

2.3.6. Test for tannin / polyphenol

To the diluted extract, 3-4 drops of 10% FeCl3 will be added, blue color will be seen for Gallic tannins and the presence of catechol tannin will turn the solution green(18).

2.3.7. Test for reducing sugar

To 0.5 mL of plant extract, 1mL of water, and 5-8 drops of Fehling's solution will be added and heated. The presence of reducing sugar will be indicated by the appearance of brick red precipitation(22).

2.3.8. Test for saponins

2 g of powdered sample was boiled in 20 mL of distilled water. 10 mL of filtrate, 5 mL of distilled water were quivered vigorously. The appearance of frothing indicated the presence of saponins(18).

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2.4. Antioxidant Activity

The antioxidant activity of G. lucidum was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method(23), DPPH reagent was prepared by adding 29.57 mg of DPPH(M.W 394.320) into 250 ml of ethanol(90%)(24) To prepare the sample solution, 25 mg of the extract was diluted to 100 ml using 90% ethanol (250 ppm). Serial dilution was performed to obtain various concentrations (250 ppm, 125, 25, 10, and 5 ppm. Subsequently, 2.5 ml of each sample concentration was mixed with 1 ml of the previously prepared DPPH reagent. The resulting solution was left to stand for 30 minutes in a dark place to allow for a chemical reaction, and the absorbance was measured at 517 nm(25).

To prepare positive control (standard solution), 250 mg of Ascorbic acid was diluted to 100 ml using ethanol (250 ppm). Serial dilution of standard was carried out to make various concentrations of (250, 125, 25, 10, and 5) ppm. Similarly, 2.5 ml of each standard concentration was mixed with 1 ml of the DPPH reagent, and the solution was allowed to stand for 30 minutes in a dark place before measuring the absorbance at 517 nm. 2 ml of ethanol was used as a blank solution.

The inhibition percentage (%) of plant extract was calculated using following equation,

$$I\ \% = \{(A_o - A_1) \div A_o\} \times 100\%$$

(Where, A_0 is the absorbance of the control and A1 is the absorbance of the extract/standard). The 50% inhibition concentration of the extract, IC50 was calculated using regression line developed from plotting a graph of scavenging percentage against the different concentrations of the extract(26).

2.5. Cytotoxic activity

Brine Shrimp lethality assay, is a preliminary assay to check cytotoxic activity(27) In this process of BSLT Artificial seawater(ph8±0.5) was made by dissolving 38 g of sea salt in 1 liter of distilled water. Water pump was used to maintain sufficient aeration and temperature was adjusted to (28±2) °C. Brine shrimp eggs were then placed in an aquarium in the absence of light to allow shrimp hatching. After 24 hours brine shrimp eggs were hatched which were left in aquarium for next 24 hours which is to allow hatchlings to become nauplii. In a test tubes of 5ml of respective sample solutions of different concentrations (500ppm, 100ppm, 50ppm, 10ppm, 5ppm and 1ppm), 5ml of salt solution and10 nauplii's were added. A test tube with only 10ml of salt solution was used as negative control. The number of alive nauplii's were counted after 24 hours and their Percentage death was calculated.(28)

$$\%\text{Death} = \frac{\text{nmber of dead nauplii's}}{\text{total number of nauplii's added}} \times 100\%$$

3. Result and discussion

3.1. Value of extract obtained by extraction (Percentage Yield calculation)

Percentage yield value of extract obtained by different method of extraction was calculated as follows;

3.1.1. Soxhlet extraction

$$\text{Yield percentage} = \frac{Extract\ obtained}{total\ amount\ of\ crude\ drug} \times 100$$

$$= \frac{18}{400} \times 100$$

$$= 4.5\%$$

3.1.2. Soxhlet followed by Maceration extraction

$$\text{Yield percentage} = \frac{\text{Extract } obtained}{total\ amount\ of\ crude\ drug} \times 100$$

$$= \frac{21.12}{400} \times 100\%$$

=5.28%

3.1.3. Maceration

$$\text{Yield percentage} = \frac{Extract\ obtained}{total\ amount\ of\ crude\ drug} \times 100$$

$$= \frac{2.60}{200} \times 100\%$$

$$= 1.3\%$$

Three distinct techniques were used to get the ganoderma extract: maceration followed by Soxhlet extraction, maceration, and Soxhlet extraction. A comparative study of the extract yield showed that the most promising approach is the combination of maceration and Soxhlet, with an extractive value of 5.28%. Soxhlet extraction method seems considerable with the extractive value of 4.5%.However, the extraction value of 1.3% indicates that the maceration approach appears to be less effective.

3.2. Phytochemical Analysis

The detection of secondary metabolites in the plant is necessary for the subsequent steps in the drug development process, including separation, isolation, and identification. the pharmacological activity of plants entirely depends upon phytochemical present in the extract .as a result of phytochemicals plant show antioxidant, anticancer, reversal of epigenetic alteration(29), antimicrobial , antipyretic, anti-inflammatory pharmacological activity(30) In this current research work, the ethanolic extract of G. lucidium was subjected to qualitative analysis to determine the secondary metabolites like carbohydrates , Alkaloid, Terpenoids, Proteins, Reducing sugar and Tannins, proteins, saponins, flavonoids and phenol.Phytochemical analysis of ethanolic extract of G. lucidium indicated that Alkaloid, Terpenoids, Proteins, Reducing sugar Tannins, saponins are present.

Table 1: Phytochemical screening of Hydroalcoholic extract of G. lucidum

Phytochemicals	Observation
Terpenoids	Present
Carbohydrates	Present
Alkaloids	Present
Reducing Sugar	Present
Proteins	Present
Saponins	Present
Flavonoids	Absent
Phenolic compounds/Tannins	Absent

3.3. Antioxidant Activity

The base line idea of antioxidant assay depends on numbers of free radicals accepted by the extract. DPPH free radical scavenging assay method is used to determine the antioxidant activity where free radical is generated by DPPH. Higher concentration of sample (i.e 250 µ/ml) react more free radicals that were generated by DPPH and decrease the absorbance in (i.e 0.05) where the lowest concentration of extract (5µ /ml) reacts with less free radical so the absorbance of lowest concentration is higher (i.e.0.20). The absorbance of reaction was measured by UV spectrometry at 517 nm. The ethanolic extract of Ganoderma shows the significant antioxidant activity and IC50 measured as 53.88±0.62 were IC50 of standard ascorbic acid were measured as 30.17. Obtained results of different concentration are presented below in the table no 1.

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Table 2: Antioxidant Activity of G.lucidum

S.N	Conc. (µg/ml)	Absorbance of standard	Negative control	%RSA of sample	%RSA of standard	IC50 of Sample	IC50 of Ascorbic Acid
1.	5	0.20	0.276	9.78	26.08	53.88 ± 0.62	30.17
2.	10	0.19	0.276	15.57	29.71		
3.	25	0.16	0.276	22.10	39.49		
4.	125	0.072	0.276	73.98	76.81		
5.	250	0.059	0.276	78.07	85.14		

A similar pattern was observed in the plot of the concentration versus percentage RSA of DPPH free radical scavenging assay. When the concentration increases the Percentage RSA value also increases upto an 1125ppm and after hence it remained almost constant. The antioxidant activity of standard seems to be a bit higher than that ethanolic extract of G.lucidum.

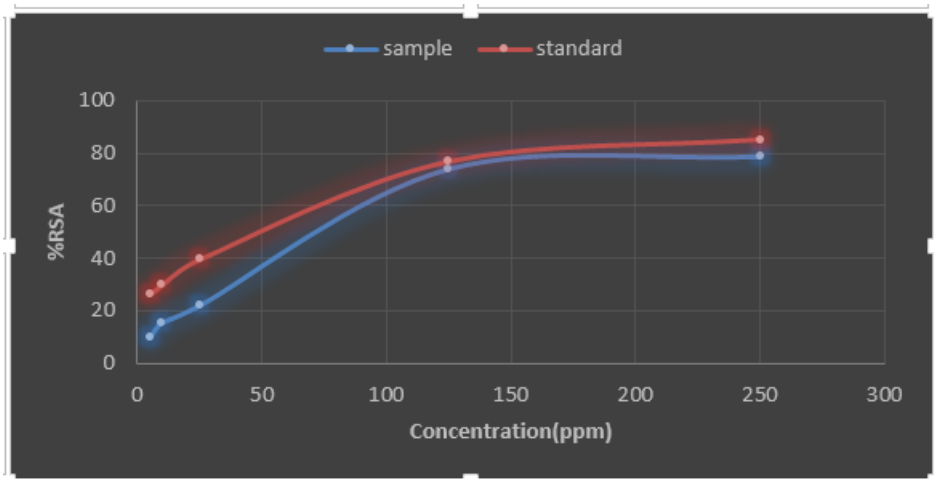

Figure 1: Graph showing %RSA of sample against concentration of extract

3.4. Cytotoxicity (Brine shrimp lethality test)

BSLT is one of the common and valid methods for a preliminary cytotoxic testing system of plant extract. Cytotoxicity of aqueous extract of G. lucidium was assessed by using the method provided by Meyer et al .in this study it is seems that when concentration increases the percentage mortality also increases in case of both test sample extract and standard. Maximum mortalities (100%) were observed at a concentration of 1000 ppm. the extent of death was higher for standard drug with LD50 of 36.67µg/ml than sample extract with LD50 of 155.89 ±1.75µg/ml ,In this study the extent of death was calculate by statistical method LD50 .when the graph is plot between concentration and percentage lethality it follows the linearlity which is shown below. The degree of lethality was directly proportional to the concentration of the extract. As shown in the figure no 2 and 3.

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Table 3: Brine shrimp lethality assay of G. lucidum (Extract)

Conc (ppm)	No. of survivor nauplii	Dead nauplii	% Death	Mean % mortality	Log Conc.	Probit value	LD50
5	10 10 10	0 0 0	0 0 0	0	0.69	0	
10	9 10 9	1 0 1	10 0 10	6.7	1	3.52	
25	9 9 9	1 1 1	10 10 10	10	1.39	3.72	
50	8 9 9	2 1 1	20 10 10	13.3	1.69	3.87	**155.89 ±1.75**
75	7 8 7	3 2 3	30 20 30	26.7	1.87	4.39	
100	5 5 6	5 5 4	50 50 40	46.7	2	4.67	
500	4 3 3	6 7 7	60 70 70	66.7	2.69	5.44	
1000	0 1 0	10 9 10	100 90 100	96.7	3	6.88	

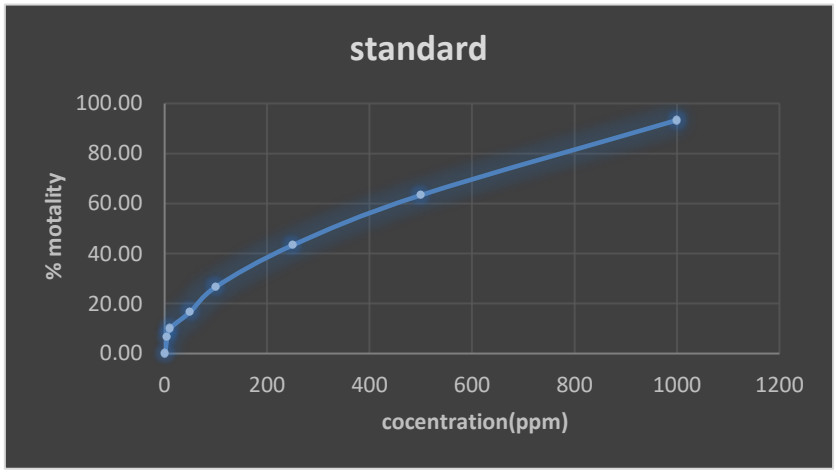

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Figure 2: percentage mortality of standard against concentration of extract

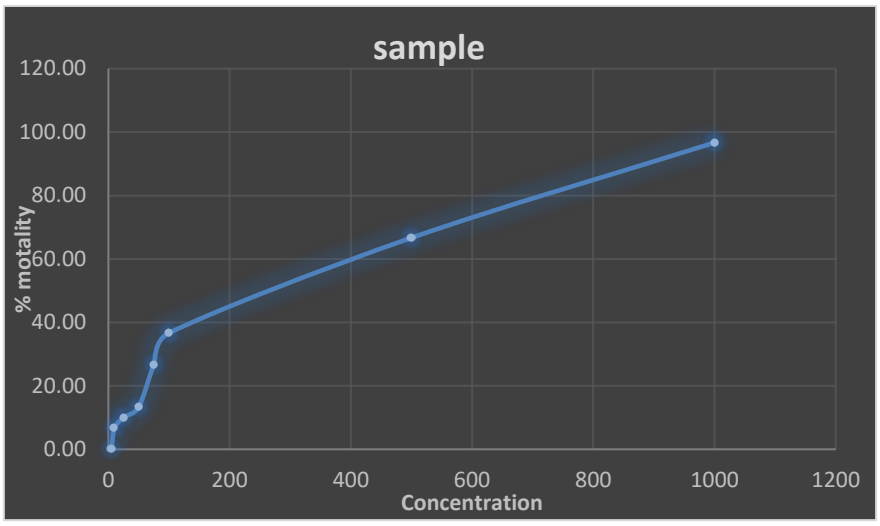

Figure 3: percentage mortality of sample against concentration of extract

4. Discussion

Distinct extraction techniques result in distinct yield values and variations in the active component patterns in the extracts(31) This research shows the higher extractive value(i.e. 5.28%) observed in maceration followed by Soxhlet extraction compared to maceration and Soxhlet alone. This result attributed to several approach that during the initial maceration step, the prolonged exposure allows for enhanced dissolution, affording the solutes more time to permeate and facilitate penetration into cellular structures and its makes more easy for the continuous and cyclic extraction process, sohxlet extraction to drag out the extract from the sample. The Soxhlet extraction method seems considerable with extractive value (4.5%).

Phytochemicals are the widely available secondary metabolites produced by plants to thrive themselves in their environments and are rich sources of therapeutic drugs. The phytochemical screening test is the most useful method to detect the biologically active compounds present into the plant's(32) .The current study of ethanolic extract of G.lucidum reveals that it contains Triterpenoids, Carbohydrates, Glycosides ,Alkaloids, Reducing sugar, Proteins,and Saponins. Although some of the Phytochemicals' such as Flavonoids, Tannins, Phenolic compounds are absent, the extract has properties on par with most of the original works conducted in this Plant. The failure of detection of Phenolic compounds might be due to deterioration occurred during shade drying of the plant. The absence of tannins can be attributed to low polarity of ethanol as compared to water(33). This Screening signifies G.lucidum is enriched with various Phytochemicals.

Free radicals, derived mainly from oxygen (ROS) and nitrogen (RNS), have gained significant attention generated in our body by various endogenous systems, exposure to different physicochemical conditions or pathophysiological states(34) When produced in excess, free radicals and oxidants generate a phenomenon called oxidative stress, a deleterious process that can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins, and In this research DPPH free radical scavenging method is used to exhibit the antioxidant level of Ganoderma lucidum(35) The Plant ethanolic extract represented an excellent ability to scavenge free radicals with IC50 of 53.88±0.62 mcg/ml Where the standard Ascorbic acid Showed IC50 value as 38.82Mcg/ml. The IC50 value of our ethanolic extract(i.e53.88±0.62 mcg/ml) was slightly lower than that of the aqueous extract in Shihabul et al. and the ethanolic extract in Sharif et ali.e(89.05 ± 3.59 µg/mLmg/ml).The lower the IC50 value, the more potent is the substance at scavenging DPPH(36) This difference could be attributed to geographical variations influencing the plant's activity. In another wordsthe findings of this study indicate that wild mushrooms collected in higher altitude regions has greater antioxidant activity.

The brine shrimp lethality bioassay was used to predict the cytotoxic activity(27). Cytotoxic effects of plant extracts represent the medicinal and pharmacological activities of plants and it is evaluated by a widely used, easy, cheapest and available bioassay called brine shrimp lethality assay(37). In this study brine shrimp lethality assay was used to determine the cytotoxicity of Ganoderma lucidum. The ethanolic extracts tested showed good cytotoxic activity. The lethality concentration (LC50) of ethanolic was 155.89 ±1.75µg/ml as shown in the table no 3. The lethality concentration (LC50) of standard drug (5-Fluorouracil) was determine 36.67µg/ml. The observed lethality of the this study indicated the presence of potent cytotoxic and probably antitumor components of these plants, The LC50 value of our study well satisfied with the Meyer et al, theory of cytotoxicity that is "crude plant extract is toxic (active) if it has an LC50 value of less than 1000 µg/mL while non-toxic (inactive) if it is greater than 1000 µg/mL."And it is also similar to LC50 values such as 142.49 ± 5.31 g/mL of ethanolic extract, 295.801mcg/ml of Methanolic extract. However in

context of solvent used Ether extract and Chloroform extract of ganoderma lucidum, LC50 values are 949.881 mcg/ml and 367.282 mcg/ml (38).clearly indicated Ethanolic extract shows better cytotoxic activity. Our ethanolic extract's LD50 value 155.89 ±1.75µg/ml was found to be similar to the aqueous extract's LD50 of 142.49+_ 5.31, as reported by (Islam et al.).The observed LC50 value of extract confirmed the Plants Cytotoxicity activity and indicated presence of potential antitumor molecules.

5. Conclusion

The lethality assay of *G. lucidum* was positive which indicates the plant may have the presence of biologically active compounds with the ability to fight or treat cancer disease. Antioxidants present in *G. lucidum* after isolation can be used to decrease the rate of mutation as well as block the development of tumors by scavenging free radicals produced in body by oxidative stress as test for antioxidant activity was positive for plant extract. Although the *G. lucidum* extract possess many pharmacological activity observed to be moderate. *G. lucidum* has excellent biomedical and pharmaceutical properties and in future it can be used as a good natural resource to develop novel drugs to treat life threatening diseases and disorders.

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Conflict of Interest Statement: All authors have nothing else to disclose.

	Anil Bhusal*(Corresponding Author) Pharmacist, B- pharm Department of Pharmacy, CIST College (Affiliated to Pokhara University), New Baneshwor, Kathmandu, Nepal
	Santosh Dhungana Pharmacist, B- pharm Department of Pharmacy, CIST College (Affiliated to Pokhara University), New Baneshwor, Kathmandu, Nepal
	Sujan Sharma Pharmacist, B- pharm Department of Pharmacy, CIST College (Affiliated to Pokhara University), New Baneshwor, Kathmandu, Nepal
	Suraj Dahal Pharmacist, B- pharm Department of Pharmacy, CIST College (Affiliated to Pokhara University), New Baneshwor, Kathmandu,Nepal
	Krishna Neupane Clinical pharmacist , M-pharm Department of Pharmacy, CIST College (Affiliated to Pokhara University), New Baneshwor, Kathmandu, Nepal

Sajan Maharjan* (supervisor)
Lecturer ,M-pharm
Department of Pharmacy, CIST College (Affiliated to Pokhara University), New Baneshwor, Kathmandu, Nepal