Nepal Journal of Biotechnology. Dec. 2019 Vol. 7, No. 1: 30-38 DOI: https://doi.org/10.3126/njb.v7i1.26948

ORIGINAL RESEARCH ARTICLE

Evaluation of Phytochemical, Antimicrobial, Antioxidant Activity and Cytotoxic Potentials of *Agave americana*

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Abstract
Ethnomedicinal plants are being used as a source of medicine from ancient time but they lack the proof of modern scientific evidence for their effectiveness. This study focuses on the evaluation of phytochemical, antimicrobial, antioxidant properties of one of the ethnomedicinal plant *Agave americana* from Dhulikhel region of Nepal. The plant extract was prepared using solvent-based warm soxhlet extraction from the leaves of the plant and antimicrobial activity against six different non-resistant clinical isolates of bacteria (*Staphylococcus aureus, Shigella, Klebsiella pneumoniae, Escherichia coli, Bacillus thuringiensis, and Salmonella paratyphi)* was evaluated using agar disc diffusion method along with qualitative analysis for presence/absence of phytochemicals. Antioxidant activity was measured by DPPH assay and the cytotoxicity was evaluated using MCF-7 (human breast adenocarcinoma) cancer cell. Presence of phytochemicals like alkaloids, flavonoids, reducing sugars and saponins were detected in the plant extract. The extract was found to show some level of antimicrobial activity against *Staphylococcus aureus, Escherichia coli* and *Bacillus thuringiensis* at 50, 100 and 200 mg/ml. The IC50 value of the extract was found to be 7.68 µg/ml. The extracts of *Agave americana* showed 50 % cell-death of MCF-7 in 12 h at 5 µg/ml. Although this study provided some scientific evidence for the medicinal value of *Agave americana*, further studies are still needed for the detailed evaluations of every molecule present in this plant along with screening in larger geographical area of Nepal.

Keywords: Antimicrobial Agents; DPPH; Phytochemical; Cytotoxicity; MCF-7

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Introduction
Medicinal plants have always been a source of treatment of diseases since ancient time [1]. Even in the modern era, medicinal plants are still being used as the primary method of disease treatment [2]. In fact, there is the whole system of medicine called Ayurveda in the Indian subcontinent region which uses medicinal plants as both complementary and alternative medicine and is being popular in other continents too [3]. There are countless literature reviews exhibiting the fact that the large group of compounds in modern medicine are derived from the medicinal plants [4] and some argue that except in the fields of infectious diseases and emergency aids the modern medicine is largely palliative rather than curative [5].

Agave americana was selected in this study which had previously shown scientific evidence of efficacy against cancer cell lines. In one of the study, steroidal saponins from *Agave americana* showed cytotoxic activity against HL-60 cell line [6]. Breast cancer is one of the major health global health problems worldwide and breast cancer cell lines are being drug-resistant [7, 8, 9]. Previous researches done in extract of *Agave americana* reveals its antioxidant potential [10] which hints the possibility of using *A. americana* as an anticancer drug against breast cancer. This study focuses on the cytotoxic potential of *A. americana* extracts rather than truly evaluating anticancer properties and the substance that are cytotoxic could be a potential anticancer agent. Six different clinical isolates viz., *Staphylococcus aureus, Shigella, Klebsiella pneumoniae, E. coli, Bacillus thuringiensis, Salmonella paratyphi* from Kathmandu University School of Medicinal Science (KUSMS) were chosen for antimicrobial screening, these microbes cause common human diseases like sinusitis, bacillary dysentery, pneumonia, gastroenteritis, paratyphoid fever etc., respectively. *Bacillus*

thuringiensis was also chosen as it shared many common phenotypic and genotypic properties with *Bacillus anthracis*, a causative agent for anthrax which is lethal in humans. All the bacteria were tested for resistant to broad-spectrum antibiotics in this study.

This research is an effort to test the type of phytochemicals present, antimicrobial activity, antioxidant potential and cytotoxic properties of *Agave americana* found in the Dhulikhel region of Nepal. Cytotoxic property of the extract was evaluated in MCF-7 breast cancer cell line. This research targets to generate supportive evidence for the medicinal values of the *Agave americana* especially against common microbes and breast cancer.

Materials and Methods

Sample collection

100 gm of *Agave americana* leaves were collected from premises of Department of Biotechnology, Kathmandu University, Dhulikhel in a sterile bag. The identification of the plant was done using literature surveys.

Sample preparation

The collected sample was then kept for drying in a well-ventilated dark room having a temperature of 25^{0}C for 30 days. Leaves were powdered using home grade grinder by grinding for 5 minutes.

Extract preparation

Extract of the plant was prepared using the warm Soxhlet extraction method [11] in which 6gm of the powdered sample of leaves was taken and packed individually into the manually prepared Whatman No 1 filter paper bag and kept into the thimble of the Soxhlet Apparatus (Borosil, Gujarat, India).

The extraction yield in percentage was calculated from the formula:

$$Extraction\ yield\ (\%) = \frac{weight\ of\ extract\ obtained}{Total\ weight\ of\ sample\ used} * 100$$

200 ml of absolute methanol was added through the thimble and the apparatus was operated continuously for 24 hours (8 hours at 40^{o}C regularly for 3 days) monitoring the circulation of water into the condenser. Then, the Soxhlet extracts in absolute methanol were treated with hexane to remove chlorophyll pigment by the help of the separating funnel, the process was repeated 4-5 times until the chlorophyll pigment was completely extracted in hexane and green color stopped appearing in the thimble. Petroleum ether was used to remove the fatty acid molecules to prepare the final extract. Then, drying was done in a water bath at 50^{o}C and the final extract was re-suspended in dimethyl sulphoxide (DMSO) for the use in all the assays and screening conducted in this study.

Phytochemical screening

Qualitative screening of phytochemicals viz., alkaloids, flavonoids, coumarin, saponins, glycosides, reducing sugar, tannins and polyphenols was done chemically [12, 13, 14].

Antimicrobial screening

Three different concentrations viz. 200 mg/ml, 100 mg/ml and 50 mg/ml of the plant extracts were prepared for assessing the antimicrobial activity against six different common disease-causing pathogenic non-resistant bacteria (*S. aureus, Shigella, Klebsiella pneumoniae, E. coli, Bacillus thuringiensis, Salmonella paratyphi*) using Agar disk diffusion method [15, 16] according to NCCLS (National Committee for Clinical Laboratory) Standard where MHA (Mueller-Hinton Agar) and McFarland 0.5 Standard were used and confirmation of non-resistance was done by antimicrobial susceptibility testing of microbes against broad-spectrum antibiotics chloramphenicol and tetracycline taking DMSO as negative control.

Antioxidant assay

Determination of total antioxidant activity through free radical scavenging activity was performed by DPPH (2,2-diphenyl-1-picrylhydrazyl, obtained from Sigma Aldrich, USA). Free Radical Scavenging Assay [17, 18] where L-Ascorbic acid was used as standard and IC_{50} (IC_{50} value indicates the concentration needed to inhibit a biological or biochemical

function by half) value was calculated for individual plant extract using UV-Spectrophotometer at 517 nm. Different concentration of 1 µg/ml, 2.5 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml of four different plant extracts were prepared using DMSO as solvent and antioxidant activities were calculated.

For IC_{50}, The capability to scavenge the DPPH radical was calculated using the following equation.

$$Percentage\ scavenging = \frac{A_0 - A_1}{A_0} \times 100\ \%$$

Where, A_0 =Absorbance of DPPH solution

A_1= Absorbance of DPPH along with the different concentration of extracts.

IC_{50} was calculated from linear trendline equation obtained by plotting a graph of concentration versus % inhibition.

Cytotoxicity analysis:

MCF-7 cell line was obtained from Everest Biotech Ltd., Kathmandu, Nepal. The MCF-7 (Human breast adenocarcinoma cell line) were seeded in 96 well plate at the density of 10^4 cells/well in 100 µl of culture medium and allowed for 24 hr incubation at 37°C with 5% (v/v) CO_2 for attachment and then three different concentration of the plant extracts viz. 5µg/ml, 20 µg/ml and 80 µg/ml prepared in DMSO were dissolved completely in the culture medium without disturbing the pellet. Readings were taken at 12 hr, 24 hr, 36 hr and 48 hr and the culture plates were kept at 37°C with 5% (v/v) CO_2 during the time frame. Numbers of cells were evaluated from manual observation in hemocytometer using phase-contrast microscope (Nikon Instruments Inc, Melville, NY, USA). The negative control for MCF-7 cell line was also used in which the plant extract was absent. Results were generated from three independent experiments and each experiment was performed in triplicate.

The percentage of cell cell-death for the treated sample was determined by the equation:

$$Cell\ Death\ \% = \frac{No\ of\ dead\ cells}{Total\ no\ of\ viable\ and\ dead\ cells} * 100$$

Results

Extraction yields

The extraction yield of *Agave Americana* from 6 gm of powder was 16.92 % (Table 1) as calculated according to the method followed by Zhang et.al. [19].

Table 1 Extraction Yield Calculation. The extraction yield was calculated to evaluate the recovery rate from *Agave americana*.

Plant used	Weight of sample(gm)	Weight of extract (gm)	Extraction yield %
Agave americana	6.0030	1.0155	16.92

Phytochemical screening

The preliminary phytochemical screening of methanol solvent extract shows the presence of alkaloids, flavonoids, reducing sugars and saponins whereas; it shows the absence of tannins, polyphenols, coumarin and glycosides (**Table 2**).

Antimicrobial assay

The susceptibility test for the antibiotics showed that all the bacteria used in the experiment are susceptible to antibiotics thus are non-resistance strains as shown in Table 3

Table 2 Results of the qualitative phytochemical screening. The extract of *Agave Americana* was analyzed for the presence of alkaloids, flavonoids, tannins, polyphenols, reducing sugars, coumarins, saponins and glycosides.

Phytochemicals	Alkaloids	Flavonoids	Tannins and Polyphenols	Reducing sugars	Coumarins	Saponins	Glycosides
Present(+) or Absent(-)	+	+	–	+	–	+	–

Table 3 Zone size interpretative standards for selected antimicrobial disks and test for resistance.

Agent	ZONE OF INHIBITION (mm)								
	Resistant[a]	**Intermediate**[a]	**Susceptible**[a]	***S. aureus***	***Shigella***	***K. pneumoniae***	***E. coli***	***B. thuringiensis***	***S. paratyphi***
Chloramphenicol[a] (30 µg Disk) (Positive Control-1)	≤12	13-17	≥18	25± 0.50	26.3±0 .76	22.5± 0.50	21.3±1 .53	26± 0.50	23± 0.50
Tetracycline[a] (30 µg Disk) (Positive Control-2)	≤14	15-18	≥19	25± 1.00	23.8±0 .76	22± 0.50	20.8±0 .76	25.1±1 .04	23.1± 1.04
DMSO (Negative Control)	NA	NA	NA	0± 0.00	0± 0.00	0± 0.00	0± 0.00	0± 0.00	0± 0.00

[a]*Source: National Committee on Clinical Laboratory Standards (NCCLS), 1998.*

as ZOIs (Zone of Inhibitions) shown by all the microbes were greater than 18 mm in diameter. Also, the test was done for DMSO which shows that DMSO used as a solvent for the extract does not inhibit the growth of the bacteria used. Chloramphenicol and Tetracycline were used as standard antibiotics to test the non-resistance nature of clinical isolates. Values are means (±SE) zone of inhibition (ZOI) in mm for antibiotics as the positive control and DMSO as the negative control are presented in the **Table 3** (n=3, P <0.05).

The extract of *Agave americana* shows antimicrobial activity in a dose-dependent manner against six different non-resistance microorganisms as shown in **Figure 1**.

Results from **Figure 1a** shows that *Staphylococcus aureus* is mildly susceptible to the extracts of *Agave americana* showing ZOI of 9 mm, 8 mm and 7 mm at the concentration of 200 mg/ml, 100 mg/ml and 50 mg/ml, respectively. Results from **Figure 1b, 1c** & **1f** shows that *Agave americana* did not show any antimicrobial activity against *Shigella, Klebsiella pneumonia* and *Salmonella paratyphi*, respectively. Results from **Figure 1d** shows that *Agave americana* seem to be mildly inhibitory at 200 mg/ml showing the ZOI of 9 mm, 8 mm and 7 mm at the concentration of 200 mg/ml, 100 mg/ml and 50 mg/ml, respectively. Results from **Figure 1e** shows that *Agave americana* is mildly inhibitory against *Bacillus thuringiensis* showing ZOI of 9 mm, 7 mm and 6 mm at concentration of 200 mg/ml, 100 mg/ml and 50 mg/ml, respectively.

DPPH free radical scavenging activity

Absorbance in 517 nm was taken for calculation of % scavenging activity of the *A. americana* extract using DPPH as control and Ascorbic acid as an analytical standard. Graph (**Figure 2**) was plotted using concentration range against percentage (%) scavenging activity and then linear trendline equations were obtained from the graph (**Table 4**) to calculate IC50 value.

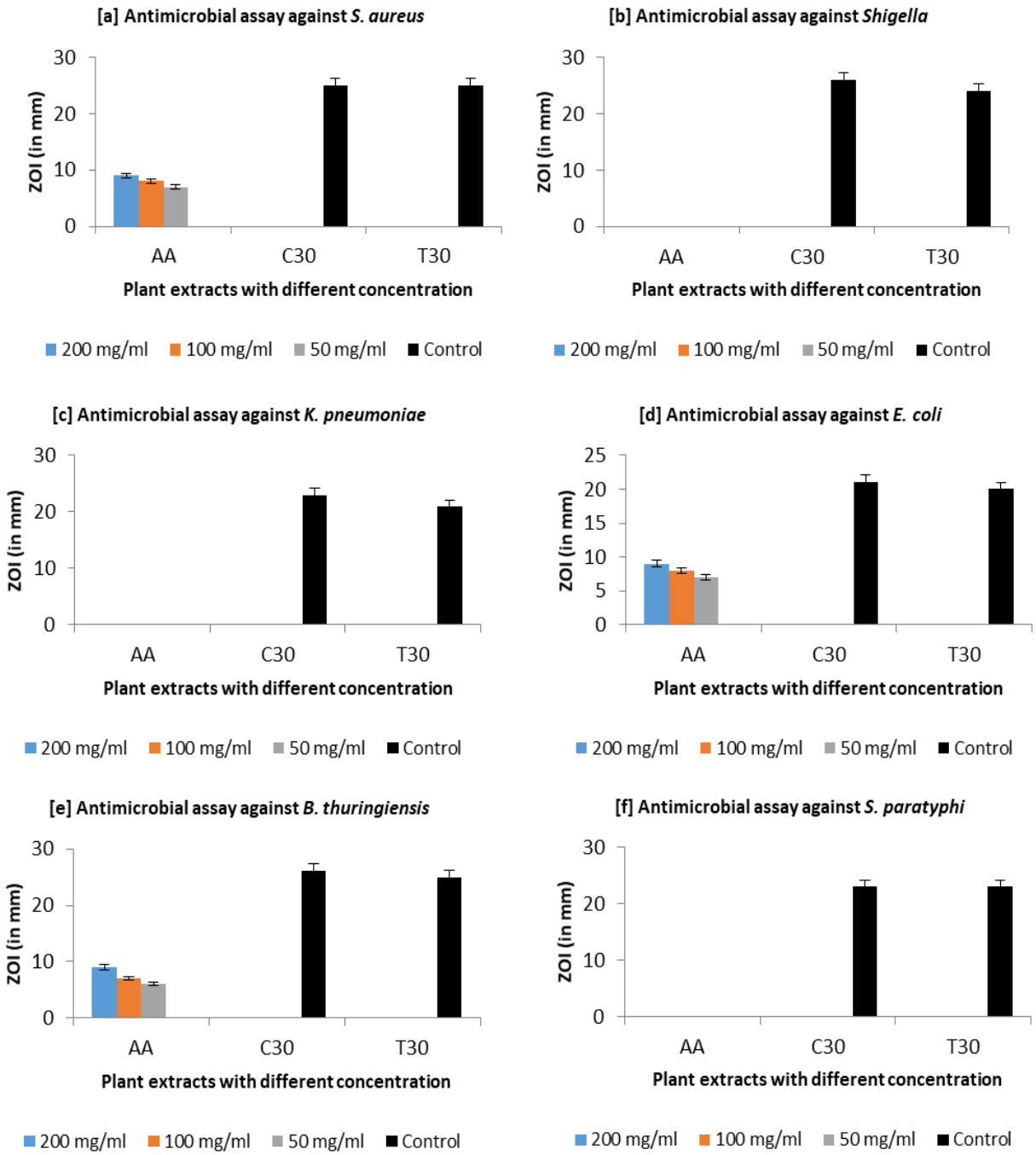

Figure 1 Graphical comparison of antimicrobial activity of A. americana extract against different clinical isolates. Figures a, b, c, d, e and f represent ZOI (Zone of Inhibition) of different concentration of the *A. americana* extracts on the individual clinical non-resistant isolates viz., *Staphylococcus aureus*, *Shigella*, *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus thuringiensis* and *Salmonella paratyphi* respectively. Different concentrations of plant extracts at 200 mg/ml, 100 mg/ml and 50 mg/ml were used for antimicrobial assay and standard antimicrobial discs were used as the positive control to ensure that microbes were not resistant to the antibiotics and false negative result about the extract's efficacy could be prevented. In the **figure AA** represents *Agave americana*. Similarly, C30 and T30 stand for Chloramphenicol 30 µg discs and Tetracycline 30 µg discs respectively. All the experiments were performed in triplicates and the error bar represents maximum of 5% error in independently performed experiments (n=3).

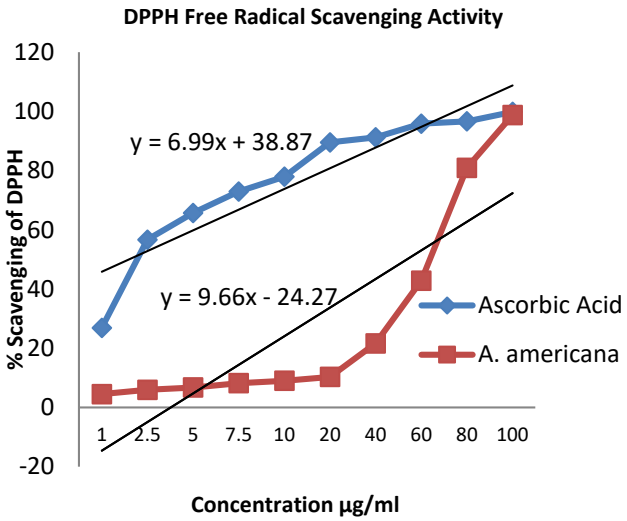

Figure 2 DPPH Free Radical Scavenging Activity of A. americana. The graph shows antioxidant assay of *A. americana* extract in comparison to ascorbic acid as the standard.

Table 4 Linear regression trendline of the extract with calculated IC50 Value. Linear regression trendline equations were obtained from **Figure 2** and IC50 values were calculated accordingly

Sample	Regression Trendline Equation	IC50 Value
Ascorbic acid	y = 6.99x+38.87	1.592 µg/ml
Agave americana	y = 9.66x-24.27	7.68 µg/ml

The IC50 value of ascorbic acid as standard was 1.592 µg/ml and the IC50 value of *Agave americana* was 7.68 µg/ml (**Table 4**).

Cytotoxicity Analysis:

The extract of *A. americana* showed cytotoxicity against MCF-7 cell line to some extent (Figure 3). Extract that shows cytotoxicity with less concentration in short time can be considered as having robust cytotoxic properties. Our experiments reveals that the MCF-7 Cell line as negative control was 100 % viable even after 48 hr thus giving the percent apoptosis a value of zero in **Figure 3**. Previously published data from Lamichhane et.al. from our laboratory also reveals that count of breast cancer cell lines does not get affected for 72 hr as a negative control [12].

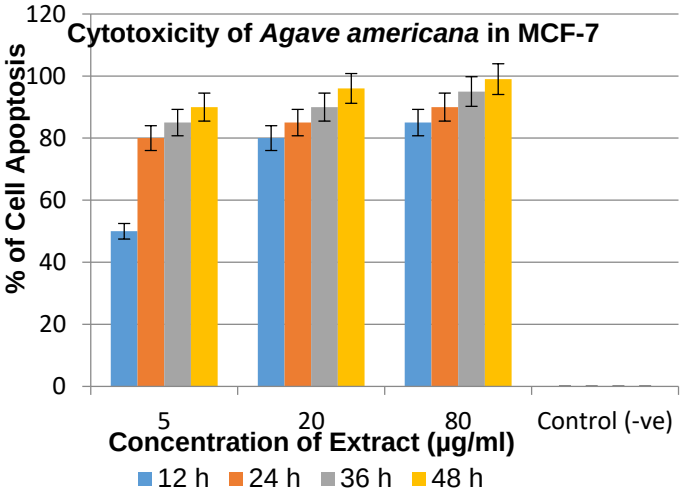

Figure 3 Comparison of percentage cell-death shown by different concentrations of the extract. The Figure represents percent cell-death of MCF-7 shown by extract of *Agave Americana* at a varying concentration of 5 µg/ml, 20 µg/ml and 80 µg/ml. All the experiments were performed in triplicates and the error bar represents the standard deviation of independently performed experiments (n=3).

Extracts of *Agave americana* showed 50 % cell-death of MCF-7 in 12 h at 5 µg/ml concentration and 85 % cell-death in 12 h at 80 µg/ml as shown in **Figure 3.** In 48 h, 80 µg/ml and 5 µg/ml of *Agave americana* extract showed 99 % and 90 % cell-death respectively.

Discussion

There is a good recovery rate from the warm Soxhlet extraction method followed as shown in **Table 1** indicating that the extracts may contain phytochemicals that can be imputed to their biological activities [20]. The phytochemical analysis as shown in **Table 2** confirms the presence of phytochemicals like alkaloids, flavonoids, reducing sugars, and saponins which may in term be responsible for the antibacterial, antioxidant and anti-cancer activities. The clinical isolates that were used in this study were tested for their non-resistance against broad-spectrum antibiotics Chloramphenicol and Tetracycline as shown in **Table 3** to prevent false-negative result and DMSO was used as a negative control as it does not exhibit any anti-microbial activities [21]. Since compounds from the natural sources are

considered as a safer option than the synthetic ones so these natural compounds can be used and explored for the treatment of the various medical conditions [22].

From **Figure 1** it can be perceived that the extract of *Agave americana* shows a certain level of antimicrobial activity against *S. aureus, E. coli* and *B. thuringiensis*. The low level of antimicrobial activity may be due to the presence of antimicrobial compounds in low concentration. If the pure compound responsible for the antimicrobial activity can be identified and isolated from the extract then the compound can show a higher level of inhibitory action.

The potent antioxidant activity in a dose-dependent manner was evaluated from the plant extract of *Agave americana* as presented in **Figure 2** and **Table 4**. From the observation, the antioxidant activity of *Agave americana* cannot be claimed strong. The lower antioxidant activity may be affected by geographical locations as some studies revealed that the same plant from different geographical regions may possess variation in compounds and thus process variation in antioxidant activities [23-25]. Hence, further screening of *A. americana* in broader geographical regions should be done to confirm the antioxidant nature.

The data in **Figure 3** shows that *Agave americana* has good cytotoxic activity against MCF-7 breast cancer cell line which can be attributed to the phytochemicals present and antioxidant activity of the plants. 5 µg/ml extract of *Agave americana* showed 50 % cell-death of MCF-7 in 12 h whereas, 20 µg/ml of the extract showed more than 80% cell-death of MCF-7 in 48 hr. The results on cytotoxic activities shown here are first of its kind for *A. americana* found in Nepal. Further research is warranted to study the molecular mechanism of their potential anti-cancer activity for the development of anticancer drugs.

Conclusions

Various literature reviews in *Agave americana* and the results obtained from this study generates some supportive evidence about its use as an ethnomedicinal plant. This study tries to explain the reason behind the use of *A. americana* from ancient times in treating diseases. The phytochemical, antimicrobial and antioxidant assays from the extract of *A. americana* show the presence of promising molecules that may be used in treating diseases.

New sources of antibiotics are currently needed in a pursuit to counter the emerging resistant strains of microbes. Also,new anticancer drugs are needed to counter the drug resistance shown by cancer cells. The ethnomedicinal plants like *A. americana* can be sources of these new molecules thus the quest to explore the initial properties of these ethnomedicinal plants should never be put to an end. Evaluation of cytotoxic properties in the extract of *A. americana* is promising which may lay a foundation to research new anti-cancer molecules.

Although the study can be a piece of scientific evidence for the properties and the use of ethnomedicinal plants like *A. americana* in terms of its antimicrobial, phytochemical, antioxidant and cytotoxic properties for pharmacological purposes but further detailed studied are still mandatory for us to elucidate a mechanistic way regarding how the individual molecules in these kinds of ethnomedicinal plants behave in-vitro and in-vivo.

Competing interests

The authors declare that they have no competing interests.

Funding

Funding for the project was entirely done by the Department of Biotechnology, Kathmandu University, Nepal.

Acknowledgments

We are grateful to the taxonomist for the identification of plant species and Prof. Dr. Tika Bahadur Karki, Head of Department of Biotechnology, Kathmandu University, Nepal for allowing us to undertake this project. We would like to thank Mr. Sandeep Adhikari, MS by research student for helping us with Cytotoxicity analysis and Mr. Basant Lamichhane, MS by research student for giving

their views in selecting medicinal plants. We would also like to appreciate Bethanie Rammer, Managing Editor at African Journal of Laboratory Medicine, United States and Dr. Ulrike Olszewski of Ludwig Boltzmann Gesellschaft, Austria who gave their valuable time and suggestions in order finalize this research article.

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