ORIGINAL RESEARCH ARTICLE

Study on Phytochemical, Antibacterial, Antioxidant and Toxicity Profile of *Viscum album* Linn Associated with *Acacia catechu*

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Abstract

This study focuses on antibacterial, antioxidant and toxic potentials of *Viscum album* Linn, commonly known as European mistletoe associated with *Acacia catechu* (Khayer in Nepali). Methanol extract of the aerial parts of the Mistletoe was prepared by cold percolation method. The resulting extract was simultaneously subjected to phytochemical screening; anti-microbial activity; anti-oxidant potential and Brine shrimp toxicity test. The major biologically active phyto-constituents observed were alkaloids, glycosides, saponins, polyphenols, flavonoids, tannins, terpenoids and cardiac glycosides. Upon antibacterial activity screening, the plant extract was found to be highly effective against *Pseudomonas aeruginosa* with the zone of inhibition 16±1mm compared to 17±1mm of chloramphenicol (50 mcg). The antioxidant activity as EC_{50} value by DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity was found to be 1.58 mg/ml while the ferric reducing capacity was measured to be 282.83±19.55 mg $FeSO4.7H_2O$ eqvt/g dry wt. of the extract during Ferric Ion Reducing Antioxidant Power (FRAP) Assay. The LC_{50} value for Brine Shrimp Toxicity Assay was found to be 31.62 ppm. This study shows the medicinal value of the mistletoe associated with *Acacia catechu*. Further meticulous analysis of this plant might lead to identification of active biomolecules effective as drugs for various ailments.

Key words: alkaloids, glycosides, polyphenols, flavonoids, tannins, terpenoids, toxicity.

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Introduction

Viscum album Linn, commonly known as European mistletoe is a semi-parasitic shrub, growing on large variety of woody trees especially on their stems and branches [1]. The plant is distributed along sub-tropical and temperate Himalayas at an altitude of 900-2100m [2]. The most common host plants include *Salix, Populus, Acer, Malus, Crataegus, Prunus, Sorbus, Abies*, and *Pinus* [3].

Viscum album in Nepal has been used as a medicinal herb since very long. Traditionally the paste of mistletoe bark is applied to relieve muscular swelling, boils and wounds and even in the management of joint dislocation; the berries act as laxative, tonic, diuretic and aphrodisiac while the plant has been used in complications like enlargement of spleen and tumors [2, 4]. The plant has also been used in case of epilepsy, vertigo, anxiety, exhaustion and hypertension [5]. Bussing (2004) has compiled wide use of this plant in various countries as: In ancient Greek in spleen diseases and complications associated with menstruation while in Argentina; mistletoe has been used as sedative and as stabilizer in bone fractures; in India the mistletoe tea in diabetes; in Africa in treatment of various stomach troubles of children including diarrhea. The German Commission E Monographs, a guide to herbal medicine approved the use of the plant in rheumatism and tremor diseases. The evergreen mistletoe was regarded as a symbol of fertility and good luck and thus was believed to avoid any fertility problems [7].

Despite the long traditional use of this herb in medicine, the chemical and biological study of this plant has only been initiated lately. The active chemical constituents reported from the mistletoe are Glycoproteins- mistletoe lectin I, II, and III; protein-viscotoxin; polysaccharides- galacturonan, arabinogalactan and alkaloids [5]. Recently two novel amino-alkaloids namely 4,5,4′-trihydroxy-3,3′-iminodibenzoic acid and 4,5,4′,5′-tetrahydroxy-3,3′-iminodibenzoic acid have been isolated and characterized [8]. Other classes of secondary metabolites from *Viscum album* are flavonoids, phenylpropanoids, triterpenes, phytosterols, cyclic peptides and cyclitols [6]. Being a parasitic plant, the phenolic content and the antioxidant capacity of mistletoe have been found to vary depending upon the host trees and the time of harvest [9,10]. New compounds have been also reported in mistletoe growing on different hosts [11].

The organic solvent fraction of mistletoe extract from its twigs and leaves showed good antimicrobial activity against both gram positive and gram negative bacteria [12, 13] .The hexane extract of the plant has shown anti-fungal activity against *C. albicans* as well [14].

Though the plant contains various cytotoxic toxins there are no reported toxicity to humans. Some studies have shown that direct ingestion of the plant parts especially berries might result in mild effects like nausea, vomiting, bloody diarrhea and shock induced hypertension [6] Application of recombinant mistletoe lectins are reported to cause some sort of reversible hepatotoxicity [15].

This plant is in high demand by the traditional healers and herbal companies for manufacturing various medicine based on this herb. In Nepal herbal drug manufacturers use this plant as an important constituent in various formulations. However the exact chemical compounds or the proven therapeutic applications of this plant have not been stated anywhere. Since this plant is parasitic in nature it has been stated that the host plant also plays important role in the active biomedical content of this plant. So, this study of *Viscum album* associated with *Acacia catechu* is unique in itself. The aim of this study is to identify active biomedical components of this plant which is associated with *Acacia catechu* and to assert any associated toxicity. We carried out phytochemical analysis of this plant which was followed by antibacterial activity screening, estimation of anti-oxidant capacity and toxicity analysis.

Materials and Methods

All the procedures were carried out in Natural Products Chemistry Laboratory, Faculty of Science, Nepal Academy of Science and Technology, Lalitpur, Nepal.

Plant Materials:

The aerial parts of *Viscum album* Linn. associated with *Acacia catechu* were collected from Sarlahi District, Nepal (1,000 - 3,300ft). It was shade dried and crushed into powder using a crusher. The resulting powder was stored in plastic bag for further work at room temperature.

Extraction of phytochemicals:

For extraction of phytochemical compounds, cold percolation method followed by intermittent sonication was followed. First the solvent methanol was added in the ratio of 8:1 (plant powder weight in gram) and shaken well then kept on stand for 3 days at room temperature. From day four onwards, the resulting solution was subjected for intermittent sonication (Rocker Scientific Co. Taiwan) - a continuous cycle of sonication at 20 kHz at 60˚C for 15 minutes followed by 2 hour standing at room temperature, till day six. On day six, the supernatant was slowly poured into the round bottom flask through cotton plug filtration which was then evaporated at reduced pressure in rotatory vacuum evaporator (Hanshin Scientific Co., Korea). Resulting dry extracts were then sealed in small glass containers and stored at 4˚C until use.

Phytochemical Screening:

The procedures for phytochemical screening has been followed in reference to the book "Phytochemical Techniques" and the research paper [16, 17]. Mayer's Test and Dragendorff's Reagent Test were employed for detection of alkaloids while carbohydrates and cardiac glycosides were detected with Molisch's Test and Fehling's Test. Proteins and amino acids were tested by applying Biuret Test and Ninhydrin Test. Liebermann-Burchard's Test gave results for presence of Phytosterols. Ferric Chloride Test, Alkaline reagent Test and Magnesium-Hydrochoric acid Reduction Test detected phenols and tannins. Bramer's Test was used for specific detection of tannins. Liebermann's Burchard test and Salkowski's Test detected terpenoids. Shinoda Test for flavonoids and Kellar-Killiani Test for cardiac glycosides were used. Saponins, Gums and Mucilages were also subsequently detected.

Antibacterial Screening:

The antimicrobial activity of the crude extract was studied by Agar well diffusion Technique [18].

Five standard strains of Gram negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhimurium* ATCC 14028, *Serratia marcescens* ATCC 13880 and *Pseudomonas aeruginosa* ATCC 27853) and a single standard strain of Gram positive bacterium (*Staphylococcus aureus* ATCC 25923) were used for antibacterial assay. The above mentioned strains of bacteria were obtained from National Public Health Laboratory, Teku, Nepal and Institute of Medicine, Maharajgunj, Nepal.

A standard antibiotic disc (Chloramphenicol, 50mcg) as positive control and DMSO as solvent control was used.

Determination of Anti-oxidant Activity:

DPPH (1,1-diphenyl-2-picrylhydrazyl) Free Radical Scavenging Activity:

A range of concentration varying from 0-10mg/ml of the sample was taken for the assay. A 50µl of the crude extract was added to 450µl of Tris-HCl buffer (0.05M, p^H7.4) and 1ml of 0.1mM DPPH was added to the resulting mixture and incubated in dark at ambient temperature for 30min. Absorbance value of resulting solution at 517nm was recorded [19, 20]

The free radical scavenging activity (RSA) of the samples was calculated in percentage by using the formula:

Radical Scavenging Activity (RSA) =

$$\frac{(Abs.Control - Abs.Sample)}{(Abs.Control)} \times 100$$

The EC_{50} values of each extract were calculated by using the formula given below:

EC $_{50}$ =

$$EXP\left[LN(Conc > 50\%) - \frac{(Signal > 50\% - 50)}{(Signal > 50\% - Signal < 50\%)} \times LN\frac{(Conc > 50\%)}{(Conc < 50\%)}\right]$$

Ferric Ion Reducing Antioxidant Power (FRAP) Assay:

10µl of the extract was added to 30µl of distilled water and 300µl of FRAP reagent; the resulting solution was incubated at room temperature for 5min. Then after the intensity of the blue colored complex formed was measured spectrophotometrically using Jenway UV-Vis Spectrophotometer at 593nm. The FRAP Reagent was prepared by mixing 25 ml of Acetate buffer (300mM Sodium acetate at p^H3.6) and 2.5ml of TPTZ (10mM 2,4,6-Tripyridyl-s-triazine in 40mM HCl) with 2.5ml of Ferric chloride solution (20mM $FeCl_3.6H_2O$ in distilled water). All the chemicals were prepared fresh and the FRAP reagent was warmed at 37°C before use. Then the reducing power of each extract was expressed as equivalent to that of 1mM of Fe (II) i.e. FRAP unit. All the tests were carried out in triplicates (n=3). [21- 23]

Brine Shrimp Lethality Assay:

Crude plant extract was prepared in three different concentrations of 1000ppm, 100ppm and 10ppm. 100ul of each of the extracts of each concentration were taken in wells of micro-titer plate. Freshly hatched brine shrimp (*Artemia salina*) nauplii were placed in each well at the rate of 5 in each and the plate was incubated at 22-28°C for 24hrs. Potassium dichromate ($K_2Cr_2O_7$ at 1mg/ml) was used as positive control [24].

The percentage viability of the larvae was observed and the percentage mortality was calculated as:

Percentage of Mortality (PM) =

$$\left(\frac{Live\ count\ in\ Control - Live\ count\ in\ Test}{Live\ count\ in\ Control}\right) \times 100$$

Then the LC_{50} values for each of the extracts were also calculated according to the formula given below:

LC $_{50}$ =

$$EXP\left[LN(Conc > 50\%) - \frac{(Signal > 50\% - 50)}{(Signal > 50\% - Signal < 50\%)} \times LN\frac{(Conc > 50\%)}{(Conc < 50\%)}\right]$$

Statistical Analysis

The final data were presented as mean ± standard deviation from the three independent assays. The DPPH radical scavenging activity and brine shrimp toxicity values were calculated using Microsoft Excel 2007 software while these data were exported to GraphPad Prism V.5.0 for generation of graphs and further analysis.

Results

The dried aerial parts upon extraction with methanol resulted into light brown dry powder. The yield percentage was 26.67% as shown in **Table 1**.

Table 1: Percentage Yield and Physical characteristics of the crude methanolic extract

Plant parts used	Dry wt. taken (gm)	Wt. of extract (gm)	Percentage yield (%)	Characteristics of extract	
				Color	Consistency
Stem and branches	8.51	2.27	26.67	Light brown	Powdery

The qualitative testing of bioactive phytochemicals in the methanolic extract of the aerial parts of *Viscum album* Linn. showed rich presence of alkaloids, saponins, phenolics and tannins. However, the flavonoid content in this extract was found to be very low. These phytochemical compounds are known to exert bioactive properties for medicinal plants. Other phytochemicals detected were steroids, terpenoids and cardiac glycosides as shown in **Table 2**.

Table 2: Preliminary Phytochemical Screening of methanolic extract

Active Compounds	Tests	Results
Alkaloids	Mayer's Test	++
	Dragendorff's Test	++
Carbohydrates and Glycosides	Molisch's Test	-
	Fehling Test	-
Saponin		+++
Protein and Amino Acids	Biuret Test	-
	Ninhydrin Test	-
Phenolics and Tannins	Ferric Chloride Test	++
	Alkaline Reagent Test	+++
	Mg Conc. HCl Test	-
Gums and Mucilages		-
Tannins	Braemer's Test	+++
Steroids	Liebermann-buchard's Test	+
Terpenoids	Liebermann-buchard's Test	+
	s	++
Flavonoids	Shinoda Test	-
Cardiac Glycosides	Kellar-Killiani Test	++

The anti-bacterial activities of methanolic extract of *Viscum album* were evaluated against 6 bacteria (5 gram negative and one gram positive). The results are

presented as zone of inhibition by the extract (mm in diameter) against the growth of the test microorganisms. The bacterium *Pseudomonas aerouginosa* was the most susceptible bacterium to the plant extract for which the zone of inhibition was marked to be 16mm while the rest of the microorganisms were not inhibited to significant extent, shown in **Table 3**. Chloramphenicol (50mcg) was employed as the positive control which showed significant inhibition to all the test organisms.

Table 3: Anti-bacterial sactivity of the crude methanolic extract

Plants / Bacterial strains	Zone of Inhibition (mm) with diameter of well=6mm					
	E. coli	*K. pneumoniae*	*Ps. aeruginosa*	*S. typhimurim*	*S. marcesens*	*S. aurues*
ATCC no.	25922	700603	27853	14028	13880	25525
V. album	6	6	16	6	6	6
Chloramphenicol (50mcg)	28	17	17	27	30	31
DMSO	6	6	6	6	6	6

The crude methanolic extract was then subjected to in vitro test to evaluate antioxidant activity using two different tests: DPPH Free radical Scavenging Assay and FRAP assay. During DPPH Assay the EC_{50} value of the methanolic extract of *Viscum album* was found to be 1.58 mg/ml which was only 50 times lower than that of the standard - Gallic acid as shown in T**able 4**.

Table 4: EC_{50} values of Crude methanolic extracts in DPPH Free Radical Scavenging Assay

S.N	Sample methanolic extract	EC_{50} values (mg/ml)
1.	Gallic acid	0.039
2.	*V. album*	1.58

The value of FRAP assay was expressed as mg $FeSO_4.7H_2O$ per gm dry wt. of the sample. In **table 5**, the FRAP activity value of the plant found to be 282.83±19.55 mg $FeSO_4.7H_2O$/gm dry wt. has been shown.

Table 5: Estimation of Antioxidant Activity of Crude Methanolic Extracts by FRAP Assay

Sample Methanolic Extract	FRAP activity (mg $FeSO_4.7H_2O$eqvt/gm)
V. album	282.83±19.55

In the brine shrimp toxicity assay, the degree of lethality was found to be directly proportional to the concentration of the extract.

All the brine shrimp larvae were found to be alive after 24 hours in the extract concentration of 10ug/ml while higher concentration was toxic enough to kill all the larvae before 24hrs**.(Table 6**) The plant extract showed significant toxicity which was measured to be an LC_{50} of 31.62 ppm **(Table 7).**

Table 6: Brine shrimp lethality assay

Concentration of *Viscum album* extract (µg/ml)	Total Number of Brine shrimp larvae used	No. of Brine Shrimp larvae alive after	
		24hrs	48hrs
1000	5	0	0
500	5	0	0
100	5	0	0
10	5	5	0

Table 7: LC_{50} values in Brine shrimp cytotoxicity assay

S.N.	Crude Extracts	LC_{50} ppm ± SD
1.	*V. album*	31.62 ± 0.00

Discussion

Preliminary Phytochemical screening of the aerial parts of *V. album* Linn. associated with *Acacia catechu*, especially the stem parts showed presence of rich bioactive secondary metabolites like Alkaloids, Cardiac Glycosides, Saponins, Phenolics, Tannins and Steroids. This result was in positive correlation with findings from Bussing (2004) and Kunwar (2010). These rich bioactive molecules have potential to be used in various ailments as tannins are capable of binding protein molecules which might have been exploited in treatment of diarrhea and skin bleedings by traditional healers. Anabolic steroids are known to retain nitrogen in osteoporosis and various wasting illnesses [25]. Alkaloids are known for their inherent toxicity to cells [26]. Flavonoids and phenolics are universally known as potent anti-oxidants and anti-inflammatory agents and therefore have been found useful against tumors and cancers [27]. Regulations of Na+/K+-ATPase-pumps are assisted by cardiac glycosides while saponins are well known for their immune-modulatory and anti-neoplastic effects [28, 29]. The rich presence of the phytochemicals of medical value thus proves the effective use of this plant in various formulations in ethno medicine.

The exciting results from the qualitative test of bioactive phytochemicals continued to show their strong presence in antibacterial activity assay as well. The crude extract was able to form a clear and distinct halo zone of inhibition measuring a diameter of 16mm (**Table 3**). This was quite comparable with that of the positive control used. *Ps. aeruginosa* is considered one of the most rapidly growing bacteria in its resistance to existing antibiotics [30]. This bacterium has been one of the major causes of hospital acquired infections all around the globe [31]. So, the excellent inhibition of this bacterium by *V.*

album Linn extract could lead to discovery of potent novel antibiotics. Although the results against other bacteria were not much promising, an increment of the concentration of the extract could have improved the inhibition ability. Since the extract used in this study is crude one, use of refined extract would have given sharper and better results.

DPPH-free RSA and FRAP assay exhibit anti-oxidant power based on electron transfer reactions. The progressive fading of the violet color of DPPH reagent to yellow indicates the scavenging of free radicals. Lower the EC_{50} value, greater is the anti-oxidant activity. The EC_{50} value of *Viscum album* in this study is 1.58 which describes better free radical scavenging ability of this plant. The co-efficient of determination (R^2 value) value of 77.30% as shown in **Figure 1** explains that the radical scavenging activity is in positive correlation with the concentration of the extract used. So, the RSA value can be enhanced further by increasing the purity of the extract. Similarly the FRAP activity has been calculated as 282.83±19.55 which is also very high value which indicates significant reduction potential of the extract. The polyphenols and flavonoids might be responsible for this active anti oxidant activities of this plant. This therefore provides evidence for the use of this plant in treatment of free radicals associated diseases like diabetes.

Toxicity is yet another important parameter to qualify any drug or drug formulation/preparation regarding safety. Preliminary monitoring of the toxicity of natural products has been carried out via Brine Shrimp Toxicity assay. The freshly hatched nauplii between 24-48 hours of hatching are considered highly sensitive to toxins and therefore this stage of brine shrimp larvae are used for toxicity assay. *V. album* extract was found to have LC_{50} 31.62 ± 0.00 ppm which is below 1000ppm and the standard chart explains that only those plant extracts having LC_{50} lesser than 1000ppm are practically cytotoxic. This observed toxicity might be attributed to high alkaloid content of the plant. Duke, 2010 has mentioned toxicity of the lectin fraction, viscotoxin and the juice of the plant with the LC_{50} value of 80µg/kg, o.7mg/kg, 32mg/kg of mouse while the capability of mistletoe lectins in inactivating ribosomes thereby halting protein synthesis has been discussed by Bussing, 2004. Therefore care must be taken while formulating this herb for drug preparation. Instead the cytotoxicity of this plant might be exploited for their use as anticancer drugs. However, *Viscum album* Linn should be subjected to further rigorous bioassays for confirmation of specific toxicity.

Conclusion

The *Viscum album* aerial parts associated with *Acacia catechu* showed rich presence of bioactive phytochemical constituents like alkaloids, glycosides, saponins, flavonoids and phenolics. The significant inhibition of *Ps. aeruginosa* presents this plant a promise for screening active constituent against its resistant forms. The significant toxicity of the extract demands careful analysis of the plant before use in medical formulations in one hand while in other it also directs its potential use for targeting cancer cells.

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