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RESEARCH ARTICLE

Study of Phytochemical Constituent and Biological Activities of Methanolic Extract of Rhizomes of *Neopicrorhiza scrophulariiflora* and Roots of *Rheum australe* collected from the Alpine Region of Nepal

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Abstract

Rhizomes of *Neopicrorhiza scrophulariiflora* and roots of *Rheum australe* were collected from the alpine regions of Jumla district of Nepal. The phytochemical analysis of methanolic extract of these plants revealed the presence of polyphenols, flavonoids, quinones, saponins and tannins. The total phenolic and flavonoid content in the extracts of *N. scrophulariiflora* were estimated to be 141.27±1.414 mg GAE/gm and 43.48±5.476 QE/gmrespectively and that in the extracts of *R. austral* were estimated to be 101.54±4.061 mg GAE/gm and 24.97±2.857 mg QE/gm respectively. Brine shrimp lethality test was performed with the extracts of *N. scrophulariiflora* and *R. austral* against brine shrimp nauplii and result revealed high toxicity of the extracts towards nauplii with LC_{50} value 173.78µg/mL and 257.03µg/mL respectively. *N. scrophulariiflora* showed the strongest DPPH radical scavenging activity with IC_{50} value 57.49 µg/mL in comparison to *R. austral* whose IC_{50} was determined to be 68.91µg/mL, ascorbic acid taken as standard with IC_{50} value 49.05µg/ml. In α-amylase inhibition assay, *N. scrophulariiflora* extract exhibited strong activity with IC_{50} value 140.67µg/mL while that of *R. austral* extract showed mild activity with IC_{50} value 192.88µg/mL.All the biological studies revealed that *N. scrophulariiflora* extract is more active than that of *R. austral* extract. Column chromatography of the extract of *N. scrophulariiflora* was performed and 50 % methanolic fraction was further subjected to FTIR and GC-MS analysis. The GC-MS analysis showed various compounds and among them, 13 major compounds were identified.

Keywords: *Neopicrorhiza scrophulariiflora*, *Rheum australe*, phytochemical analysis, Brine shrimp lethality, α-amylase, GC-MS

1. Introduction

In spite of its limited boundary, due to the divergence in altitude and climate, Nepal is native to varieties of medicinal plants. More specifically, the epitome of biodiversity lies in a narrow region on the southern margin of the Himalayas, the world's highest mountain ranges with elevation exceeding 8000 m [1]. The plant diversity of this region is characterized primarily by altitudinal zonation and monsoonal rainfall. Hara and co-workers have estimated there to be around 6000 species of higher plants in Nepal, including 303 species endemic to Nepal and 1957 species restricted to the Himalayan range [2,3,4]. The importance of such plant has been felt and widely used in Ayurveda, Homeopathy, Unani system, Chinese and Tibetian practices [5]. In addition to this reality, approximately 25% of modern pharmacological drugs are derived from plants or other synthetic analogous are built on prototype compounds isolated from plants [6]. These bioactive non-nutrient compounds are called

phytochemicals and are generally heterogeneous class of compounds derived from the secondary metabolites [7]. Phytochemicals are usually synthesized in different chemical configurations from primary metabolites by regulating the primary biosynthetic pathway metabolism of plant species which are based on the secondary metabolites requirements appropriate to the ecological necessity [8]. So far, about 10,000 phytochemicals have been identified and still a large percentage remains unknown. These identified phytochemicals include tannins, flavones, triterpenoids, steroids, saponins and alkaloids [9]. Several researches revealed that the primary physiological functions of phytochemicals are to serve as a plant defense mechanism against plant pathogenic pests, herbivores, UV light and oxidative stress [10].

Apart from their defensive function, phytochemicals like β-carotene, vitamin E, ascorbic acid, etc. help to scavenge reactive oxygen species and other free radicals that are responsible for various diseases including Alzheimer's diseases, diabetes, asthma, meningitis and gastrointestinal infections [11, 12]. Numerous earlier studies have reported the phytochemicals to be effective on as anti-ulcer, anti-inflammatory, anti-diabetic, anti-microbial, neuroprotective and anti-oxidant functions [13,14,15,16].Recently, the screening of chemical constituents as potential α-amylase inhibitor in medicinal plants have received more attention as therapeutic source for the treatment of type II diabetes because inhibition would decrease the absorption of glucose and consequently reduce postprandial blood glucose level[17].Phenolics and flavonoids have demonstrated the highest inhibitory activities [18]. Research have shown that Brine Shrimp Lethality test is an important tool to screen a wide range of chemical compounds for detecting a broad spectrum of pharmacological activities [19].

This paper entails two important plants growing wild in the alpine regions of Nepal. *N. scrophulariiflora*, belonging to scrophulariaceae family, locally called as Kutki or Katuki, naturally grows in wild in alpine regions between 3500 to 4800 m [20]. Its traditional uses have been reported beneficial for diabetic patient, cure cuts and wounds and against stomach problem such as colic, spasms and indigestion[21]. Another important plant under study is *R. australe*, belonging to Polygonaceae family, locally known as padamchallno, naturally grows in altitude ranging from 1800 to 2800 m in the temperate and subtropical regions of Himalayas of Nepal[22].The rootstock of this plant has been used against helminthes, bile disorder, bloated stomach, blood disorder, fractured bones, chest pain, cold, constipation, cough, diarrhea, indigestion, internal injury, joint pain, menstrual disorder, scabies and tonsillitis. In addition to these health benefits, rhizomes of this plant have been used to treat boils, bruises, bronchitis, and frost bite [23]. Methanolic extract of *R. australe* has been reported to exhibit anti-inflammatory [24],antifungal[25], antidiabetic[26], nephroprotective[27],hepatoprotecti ve[28],anti-cancer [29] and anti-oxidant activity[30]. Despite the fact that parts of *N. scrophulariiflora* collected in Jumla district, has been used for numerous health benefits and are promisingly therapeutically significant, detail assessment of its biological activities are still lacking despite 124 major phytochemicals have been isolated from its rhizome extracts [22].This paper aims to encompass phytochemical screening, Brine Shrimp bioassay, quantitative analysis of phenolic and flavonoid content, study of antioxidant activity using DPPH assay, evaluation of α-amylase inhibitory activity of methanolic extracts of *N. scrophulariiflora*and *R. australe* and GC-MS and FTIR analysis of methanolic extract of *N. scrophulariiflora.*

2. Materials and Methods

2.1 Collection and Identification of Plant Sample

The fresh plant rhizomes of *N. scrophulariiflora* and roots of *R.australe* were collected from alpine regions of Jumla district and their taxonomic identification was done from National Herbarium and Plant Laboratories, Lalitpur (Voucher details: *Neopicrorhizascrophulariiflora* (Pennell) D.Y.Hong,Gothichaur, Jumla district, 3700m, 06/19/2018, Ganesh Prd. Kumai G01 and *Rheum australe*D.Don, Gothichaur, Jumla district,3700m, 06/19/2018, Ganesh Prd. Kumai G02).Collected

samples were washed, shade dried, grounded to powder and stored in a plastic bag for further analysis.

2.2 Chemicals and Equipments

Solvents like hexane, ethyl acetate, methanol, DMSO and chloroform, all of analytical grade, were purchased from Merck and Scientific Fischer. Silica gel for column chromatography and precoated TLC plates were purchased from Merck Company. The eggs of *Artemia* salina (Brine) were purchased from local vendor. Porcine pancreatic α-amylase (PPA) was purchased from Sigma Chemical Company, USA and other chemicals like DPPH, ascorbic acid, gallic acid, quercetin, etc. were purchased from Fisher Scientific, USA. GC-MS instruments (Shimadzu 2010) of National Forensic Laboratory, Lalitpur, and FTIR instrument (IR Tracer 100 Shimazdu) of central department of chemistry, Tribhuvan University were used for the analysis of plant extracts.

2.3 Extraction

Cold percolation process was used for extraction using methanol. About 100 gram of powdered materials was kept in conical flasks with methanol for 3 days at room temperature with occasional shaking. The content was then filtered and filtrate was concentrated using rotatory evaporator. The process was repeated for 6-7 times and thus obtained concentrated filtrate was air dried to obtain solid residue. These residues were kept in vials and stored at 4 °C until further use.

2.4 Phytochemical Screening

The phytochemical screening of methanolic extracts were performed using standard protocol [31].

2.5 Biological Screening

Present work circumscribes the screening of the extracts for brine shrimp bioassay, antioxidant activity, total phenolic and flavonoid content and anti-diabetic activity.

2.5.1 Brine shrimp bioassay

The cytotoxicity test involving Brine shrimp bioassay was carried out following the standard protocol which involved the introduction of newly hatched Brine shrimp nauplii to each crude methanolic plant extract and determination of the LC_{50} values of the crude extracts in µg/mL [32].

2.5.2 Antioxidant activity

The antioxidant activity of the plant extracts was analyzed by DPPH radical scavenging assay, using the standard protocol [33]. Different concentrations (5 ppm, 10 ppm, 15ppm, 20 ppm, 30 ppm, 40ppm, 50ppm and 100ppm) solutions of ascorbic acid and methanolic extracts were made from their stock solutions and their scavenging activity were observed in terms of absorbance at 517 nm. From these absorbance values, % scavenging activity and IC_{50} values were calculated.

$$\% \text{ scavenging} = \left[\frac{(Ac - As)}{Ac}\right] \times 100$$

Where, As is the absorbance of sample solution and Ac is the absorbance of control

2.5.3 Total Phenolic content (TPC)

Total phenolic content was analyzed by Folin-Ciocalteu colorimetric method based on oxidation-reduction reactions as described by standard protocol [34]. Gallic acid was used as a standard.

2.5.4 Total Flavonoid content (TFC)

Total flavonoid content was analyzed by aluminum chloride colorimetric method as described by standard protocol [35]. Quercetin was used as a standard.

2.5.5 Anti-diabetic activity

Anti-diabetic potential of methanolic extracts were determined via α-amylase inhibition assay following the standard protocol with slight modification [36]. The undigested starch due to enzyme inhibition was detected through blue starch iodine complex detected at 630 nm.

2.6 Isolation of compounds using silica gel column chromatography

About 12 gram methanolic extract of rhizomes of *N. scrophulariiflora* was absorbed in (about) 25-gram silica gel and was loaded on to a silica gel packed column. Hexane, ethyl acetate and methanol at various

proportions were used as mobile phase. Altogether 163 different fractions were obtained which were further mixed and classified into 21 fractions on the basis of similar TLC results.

2.7 GC-MS and FTIR analysis

Column fraction (137-142) obtained at 50% methanol in ethyl acetate mobile phase was further analyzed using GC-MS instrument at National Forensic laboratory. The methanolic extract of *N. scrophulariiflora* was subjected for FTIR analysis at Central Department of Chemistry, Tribhuvan University.

3. Results and Discussion

3.1 Phytochemical Screening

Preliminary phytochemical screening revealed the presence of flavonoids, reducing sugar, terpenoids, phenolic compounds, tannins and quinones in methanolic extracts of both plants under study. In addition, glycosides were present only in methanolic extracts of rhizomes of *N. scrophulariiflora* whereas alkaloids, saponins and sterols were present only in the methanolic extracts of roots of *R. australe*. The results showed that both plants are rich in phytochemical constituents.

3.2 Biological Screening

3.2.1 Brine Shrimp Bio-assay

Newly hatched brine shrimp nauplii were exposed to different concentration of methanolic extracts and methanol. The toxicity of these extracts were evaluated by calculating LC_{50} values (µg/mL). Since, those extracts whose LC_{50} value found to be less than 1000 µg/mL are considered to be pharmacologically active[32], both methanolic extracts of rhizomes of *N. scrophulariiflora*and roots of *R.australe* were found to be active with LC_{50} value 173.78 and 257.03 µg/mL respectively. More precisely, methanolic extract of *N. scrophulariiflora* was found to be more active towards this assay. The concentration dependent increment in mortality may indicate the presence of cytotoxic principles in these extracts. This bioassay has been significantly correlated with *in vitro* growth inhibition of human solid tumor cell lines demonstrated by the national cancer institute, USA and thus this bioassay can be used as a pre-screening tool for anti-tumor drug research [37].The details of Brine shrimp bio-assay of plant extracts are shown in following table.

3.2.2 Antioxidant Activity

The antioxidant activity of plant extracts was studied as DPPH free radical scavenging activity using Ascorbic acid as a standard, following the standard protocol [32].The absorbance of various concentrations recorded at 517 nm were used to calculate % free radical scavenging activity and finally $IC_{50.}$ The % free radical scavenging activity of methanolic extracts and ascorbic acid with respect to various concentration is demonstrated as a plot in figure 1.The IC_{50} values of methanolic extract of *N. scrophulariiflora*and *R. australe*were found to be

Table 1: *Brine shrimp bioassay and calculation of LC_{50} values of methanolic extracts of Rheum austral and N. scrophulariiflora.*

Name of the Extract	z	x=logZ	No. of alive larvae(y)	n	xy	x^2	β	α	X	LC_{50}
MeOH extract of *R. a* root	10 100 1000	1 2 3 ∑x=6	8 6 3.66 ∑y=17.66	3	8 12 10.98 ∑xy=30.98	1 4 9 $\sum x^2$=14	-2.17	10.2	2.41	257.03
MeOH extract of *N.s* rhizomes	10 100 1000	1 2 3 ∑x=6	7 6 3.33 ∑y=16.33	3	7 12 9.99 ∑xy=28.99	1 4 9 $\sum x^2$=14	-1.84	9.12	2.24	173.78

Where, z = concentration in µg/mL, n = no. of replicates, LC_{50} = antilog X, x = log of concentration of the solution in µg/mL, $\alpha = \frac{\sum y - \beta \sum x}{n}$ $\beta = \frac{\sum xy - \sum x \sum y/n}{\sum x^2 - (\sum x)2/n}$ and X = (Y-α)/β

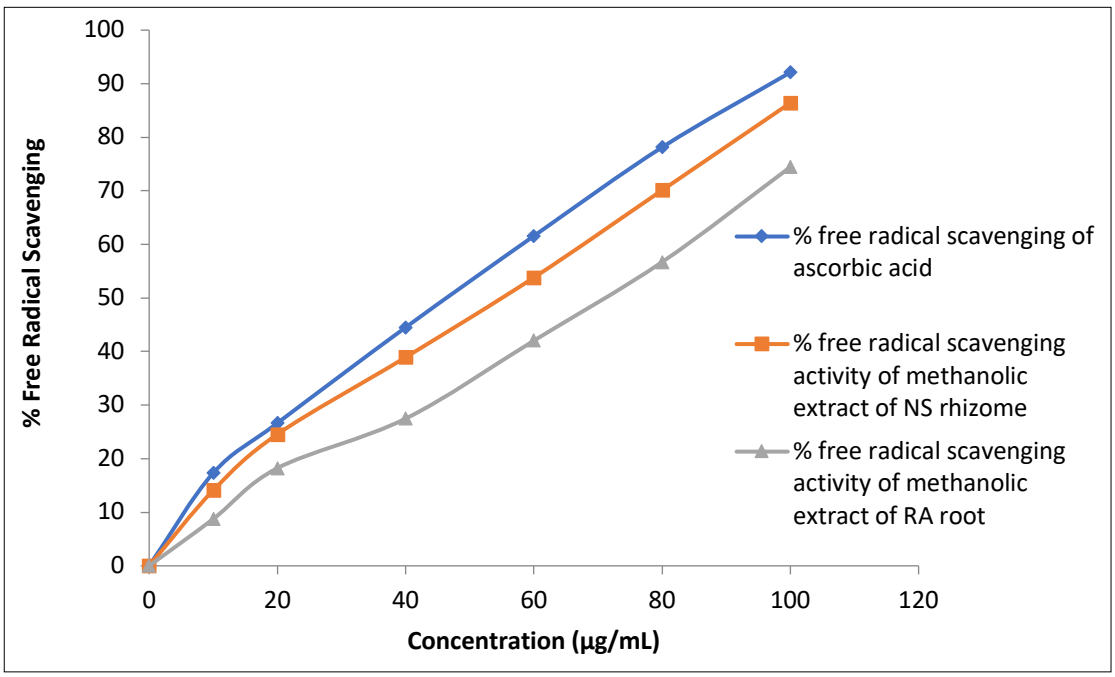


Figure 1: *A plot of % free radical scavenging activity versus varied concentration of plant extracts and ascorbic acid.*

57.49µg/mL and 68.91µg/mL respectively, which are comparable with the IC_{50} value of the standard ascorbic acid (49.05µg/mL). Thus, these extracts could be the source of potential antioxidants. Several studies have revealed that the phenolic composition of plant is linked with their antioxidant properties [38,39,40]. Polyphenols, broadly divided as flavonoids, stilbenes, lignans and phenolic acids [37,38] reacts with a free radical, resulting delocalization of the gained electron over the phenolic antioxidant and the stabilization by the resonance effect of the aromatic molecule, which cease the continuation of the free radical chain reaction [40,41].Similar type of antioxidant activity for *Picrorhizakurroa* has been reported [30]. *N. scrophulariiflora* is used heavily as a substitute for *P. kurroa*

3.2.3 Total Phenolic Content (TPC)

The total phenolic content in both plant extracts were calculated from the calibration curve using regression equation $y = 0.0142x + 0.0222$ and R^2 = 0.9985, followed by the formula C = cV/m and expressed as mg gallic acid equivalent (GAE) per gram of the extract in dry weight. The results showed that both plant extracts possessed high phenolic content. The total phenolic content present in rhizome extract of *N. scrophulariiflora* and root extract of *R. australe* were found to be 141.27±1.414 and 101.54±4.061 mg GAE/gram. Bhatt et al (2017) reported similar study in rhizome extract of *N. scrophulariiflora* and revealed the total phenolic extract to be 124.0±0.3 mg GAE.gramof the extract in dry weight [42].

3.2.4 Total Flavonoid Content (TFC)

The total flavonoid content in both plant extracts were calculated from the calibration curve using regression equation $y = 0.0015x - 0.0016$ and R^2 = 0.995, followed by the formula C =cV/m and expressed as mg Quercetin equivalent (QE) per gram of the extract in dry weight. The study revealed that total flavonoid content was found to be higher in rhizome extract of *N. scrophulariiflora* with the calculated value 43.48±5.476 mg QE/gram extract. In the similar study, the root extract of *R. australe* contained 24.97±2.857 mg QE/gram extract.

3.2.5 Anti-diabetic assay

α-Amylase inhibitory assay was performed to evaluate anti-diabetic effects of the methanolic extracts using starch-iodine method [36]. Percentage inhibition of amylase by different concentration of methanolic extracts of *N. scrophulariiflora* and *R. australe* andacarbose (standard) were evaluated and tabulated which is represented in table 2. The result revealed

that both plant extracts exhibit α-amylase inhibition assay and their inhibition activity increased with the increase in concentration of extract. The inhibition of α-amylase by plant extracts were correspondent with Acarbose, a standard α-amylase inhibitory compound. The relative comparison of % α-amylase inhibition by different plant extracts and Acarbose at different concentration is represented in figure 2.

Table 2: *α-amylase inhibition % by different concentration of plant extracts and Acarbose*

Sample No.	*N .scrophulariiflora*	*R.australe*	Acarbose
Concentration (µg/ml)	**Percentage inhibition**		
1000	84.08 ± 0.25	76.86 ± 0.22	91.60 ± 0.31
640	80.88 ± 0.23	73.70 ± 0.25	85.57 ± 0.23
320	71.42 ± 0.17	68.26 ± 0.21	76.46 ± 0.34
160	67.21 ± 0.19	63.32 ± 0.24	69.64 ± 0.65
80	58.29 ± 0.26	55.92 ± 0.32	63.69 ± 0.54
40	53.69 ± 0.20	52.30 ± 0.31	56.60 ± 0.31

(Each value is a mean of triplicate data)

IC_{50} values were obtained from the plots of % inhibition against concentration shown in (table 3). which revealed that methanolic extract of *N. scrophulariiflora* showed higher anti-diabetic property than that of *R. australe.*

3.2.6 Correlation between TPC, TFC, Anti-oxidant and Anti-diabetic activity:

It is well established fact that plant phenolics are highly effective free radical scavengers and antioxidants. Thus, there should be a close correlation between the content of phenolic compounds and anti-oxidant activity as depicted by Bravo [43].In the similar study, it was found that phenolics and flavonoids play significant role in the medicinal properties. This is manifested by strong radical scavenging activities. The discoloration indicates the scavenging potential of the sample antioxidant such as phenolic compound, especially phenolic acid and flavonoids [44]. Thus, high phenolic and flavonoid content in methanolic extracts of rhizomes of *N. scrophulariiflora* and roots of *R. australe* must correlate with its significant DPPH free radical scavenging activity and α-amylase inhibition assay. Table 3 shows total phenolic and flavonoid content in plant extracts with their antioxidant and anti-diabetic properties.

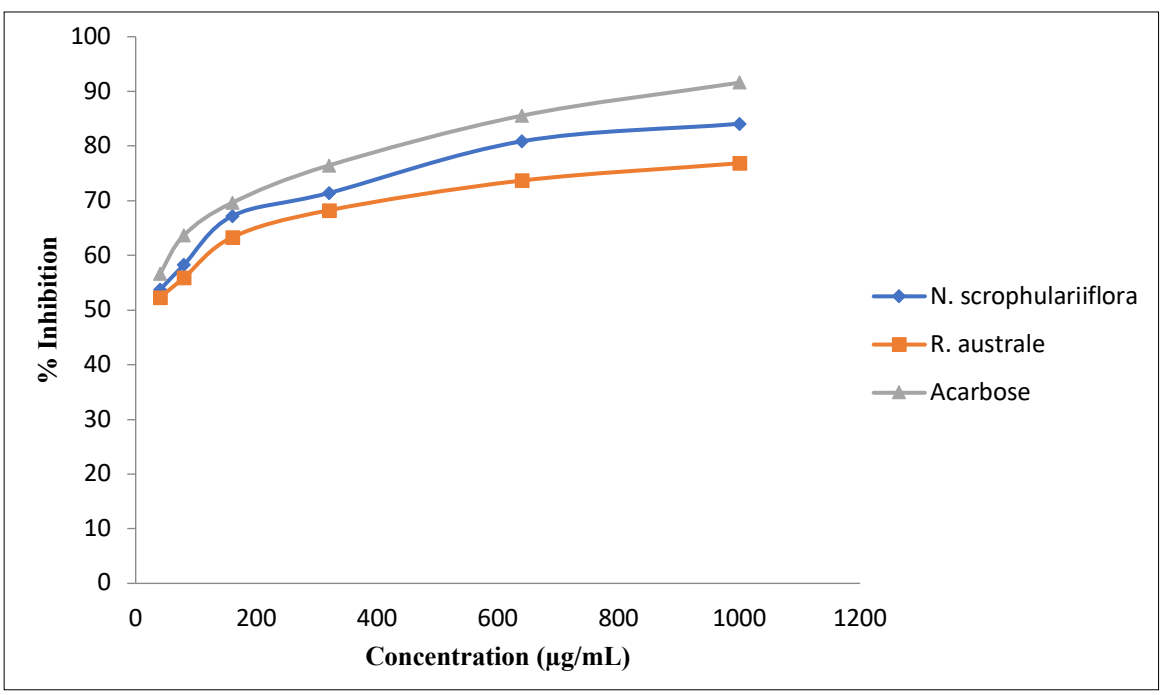


Figure 2: Comparison of α-amylase inhibition % between Acarbose, methanolic extract of *N. scrophulariiflora*and *R. australe.*

Table 3: *Correlation between TPC and TFC of plant extracts with its antioxidant and anti-diabetic properties.*

Plant Extracts	Total Phenolic content(mg GAE/gram of extract)	Total Flavonoid content (mg QE/gram of extract)	DPPH assay IC_{50} value (µg/mL)	α-amylase inhibition assay IC_{50} value (µg/mL)
Rhizomes of *N. scrophulariiflora*	141.27 ±1.414	43.48±5.476	57.49	140.67
Roots of *R. australe*	101.54±4.061	24.97±2.857	68.91	192.88

P.S. Ascorbic acid was taken as a standard for DPPH assay with IC_{50} = 49.05µg/mL and Acarbose was taken as a standard for α-amylase inhibition assay with IC_{50} = 86.12 µg/mL.

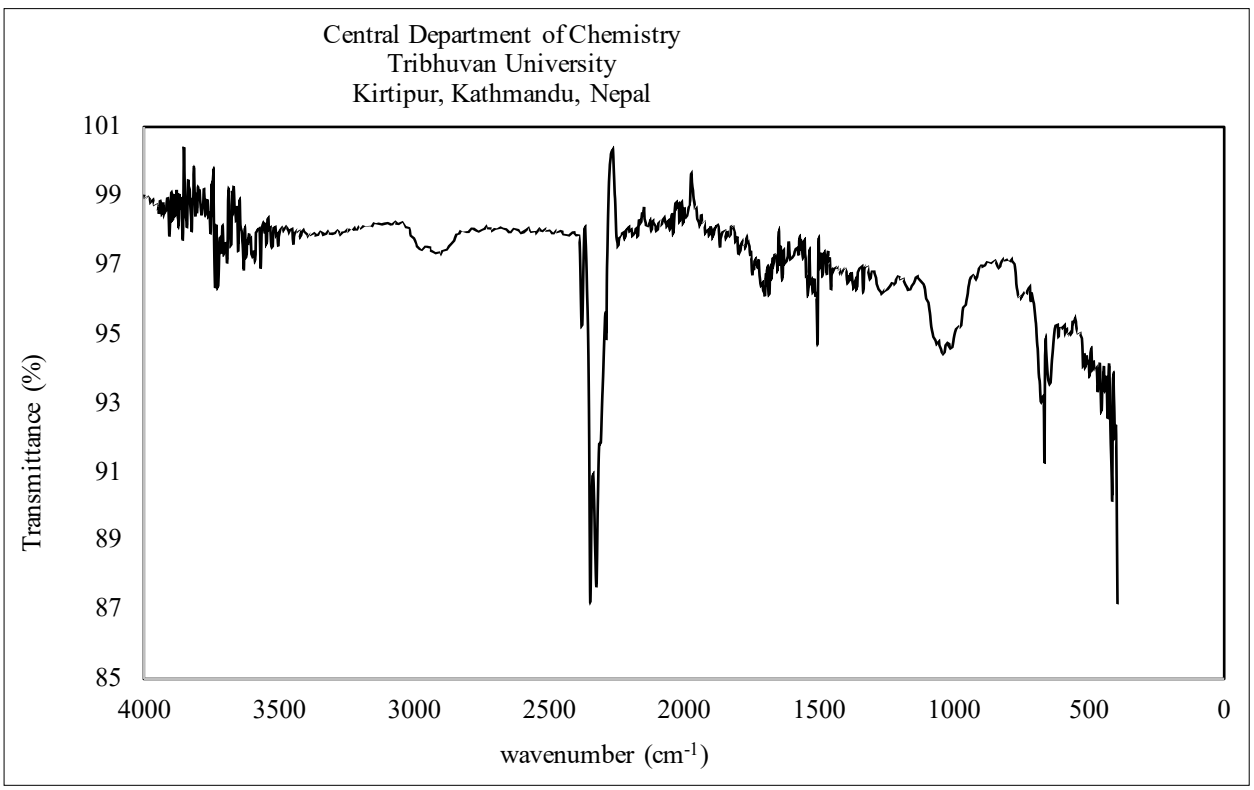


Figure 3: *IR spectrum for column fraction of methanolic extract of N. scrophulariiflora*

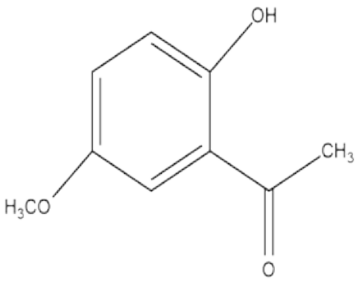
3.3 FTIR and GC-MS analysis

Column fraction(137-142) of methanolic extract of rhizomes of *N. scrophulariiflora* was further analyzed via FTIR which showed broad peak at 3200-3600 cm^{-1} indicating the presence of O-H bond (either H bonded alcohol or phenol group).In addition, a peak at 2200-2400 cm^{-1} may be due to CO_2 group from air (figure 3). The GC-MS analysis showed various compounds, among which 13 major compounds are given in the table below (Table 4).

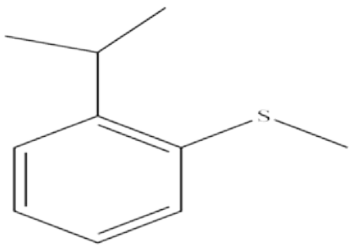
Table 4: *List of compounds detected from GC-MS analysis of N. scrophulariiflora*

SN	Name of Compounds	Area %
1	5-formyl salicyclic acid	1.25
2	cis- cinnamic acid	1.35
3	Trans-cinnamic acid	1.39
4	Phthalamide	1.47
5	Cyclohexane-1,4,5-triol-3-one-1-carboxylic acid	1.75
6	4-nitroimidazole-5-propnoic acid	4.07
7	3-hydraziano-4-methylbenzoic acid	4.16
8	2,6-bis(1,1-dimethyl)-Phenol	4.88
9	3-(1,5-dimethyl-4-pyrazoyl)-propanoic acid	5.38
10	2-nitrophenyl cinnamamide	5.84
11	t-butyl hydroquinone	6.07
12	S-methyl-2-isopropyl benzenethiol	6.70
13	1-(1,5-dimethyl-4-pyrazolyl)-propanoic acid	17.25

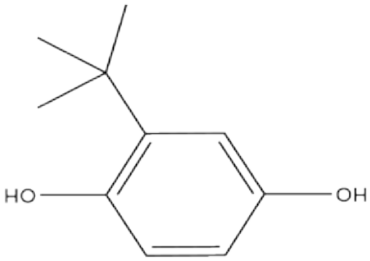
The structures of some major compounds detected via GC-MS analysis under retention time 11.79-11.97 minutes are shown below;



1-(1,5-dimethyl-4-pyrazolyl)-propanoic acid



S-methyl-2-isopropyl benzenethiol



t-butyl hydroquinone

4. Conclusion

The results from this study reveal that both plants are rich in phytochemicals such as polyphenols, flavonoids, glycosides, saponins and tannins. Both extracts contained high phenols and flavonoid constituents and their methanolic extract showed significant cytotoxicity, anti-oxidant and anti-diabetic properties. The results from these studies suggest that methanolic extract of rhizomes of *N. scrophulariiflora* contain higher amount of phenolic and flavonoid compounds and thus showed better biological assays as compared to methanolic extracts of roots of *R. australe*.GC-MS analysis of column fraction of methanolic extract of *N. scrophulariiflora* rhizomes revealed 13 compounds, 1-(1,5-dimethyl-4-pyrazolyl)-propanoic acid being the major one. In essence, both plant extracts showed major biological activities and can be the potent source of phytochemicals. However, extensive pathological and pharmacological studies along with the mechanism of action should be investigated for their in-vivo application.

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Conflict of Interest:

Authors have no conflict of Interests.

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