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Research paper

Comparative analysis on chemical composition of *Citrus limon* (L.) Osbeck and *Citrus sinensis* (L.) Osbeck leaves

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ABSTRACT

Context: Essential oils from Citrus species are considered as a rich source of bioactive compounds. ***Objectives***: The study explores the chemical constituents of *Citrus limon* (L.) Osbeck and *Citrus sinensis* (L.) Osbeck essential oils from different provinces of Nepal. ***Methods***: The essential oils were extracted by the hydro-distillation method using a Clevengers' type apparatus and their chemical compounds were identified by gas chromatography and mass spectrometry (GC-MS). ***Results***: Total 45 compounds were identified from all samples where 12, 11, and 21 compounds were from Lemon oil of Koshi, Gandaki, and Bagmati province respectively. Similarly, in the orange oil total 13, 17, and 18 compounds were identified in Koshi, Bagmati, and Gandaki province samples respectively. Based on the mass spectra of a compound, it was identified as Citronellal. This compound was found as 52.77% and 27.75% concentration in lemon of Koshi, and Gandaki provinces whereas another compound Geranial (29.37%) was dominant in Bagmati province lemon samples. Similarly, Linalyl anthranilate was dominant in orange samples with 25.88 to 44.98% concentration. ***Conclusion***: Altitude influences the composition of essential oil from lemon and orange in Nepal.

Keywords: Citrus species, essential oils, GC-MS, monoterpenes, TLC

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INTRODUCTION

Citrus essential oils are multifunctional natural products useful for food, pharmaceutical, and cosmetic industries. The most economically important genera in the family is *Citrus*, which includes the orange *Citrus limon* (L.) Osbeck and *Citrus sinensis* (L.) Osbeck, and many useful fruits (Tamokou et al., 2017). Several species are recognized for their medicinal, physiological, and pharmacological activities including antimicrobial, antioxidant, anticancer, anti-inflammatory, and hypoglycaemic activities (Kaur et al. 2023, Saeb et al. 2016). While there are several compounds unique to citrus, which are relatively rare in other plants, their individual components have also demonstrated anticancer activity against different celllines (Gyawali and Kim, 2014). The main chemical components of lemon and orange essential oils are limonene, α-terpene, γ-terpinene, α-terpineol, β-pinene, α-pinene, myrcene, linalool, citronella, β-bisaboline, caryophyllene, citral (Owolabi and Avoseh, 2018; Zeleke, 2022). The most valuable compound biosynthesized in oranges is limonene (Kim et al., 2017).

Essential oil originated from various places have a substantial impact of geography on its chemical constituents and biological functions (Owolabi & Avoseh, 2018). Environmental factors exert the influence on metabolite production of any medicinal plant (Milos et al., 2001; Zgheib et al., 2016; Morshedloo et al., 2018; Nirala et al., 2024). Such relationship between the level of secondary metabolites and external variables may reflect the degree of adaptation and variation to particular environmental features (Jaakola & Hohtola, 2010). Furthermore, soil physical and chemical properties may influence plant physiology, which can lead to differences in plant chemistry (Misal et al., 2012, Dubuis et al., 2013).

Orange and lemon are known 'suntala' and 'kagati' respectively in Nepali, whereas 'nāraṅgam' and 'Jambeera' in sanskrit from Citrus family. Plants are used in various medicinal purposes such as skin diseases, blood related problems, ulcer wounds etc (Baral and Kurmi, 2006). Leaves are applied to forehead to treat headache, jaundice, and oral thrush. Essential oils are used to cosmetic and home cleaning products. Very little is known regarding the chemical composition in the samples collected from various geographical locations in Nepal. Hence, this research was designed to identify and compare the constituents among two citrus plant leaves collected from different provinces of Nepal.

METHODS

Collection and preparation of plant extract: The leaves of orange and lemon were collected from Koshi, Bagmati and Gandaki province of Nepal during their matured period i.e. August to October 2022. Herbarium of the plants were identified by Miss Rita Chhetri, taxonomist, National Herbarium and

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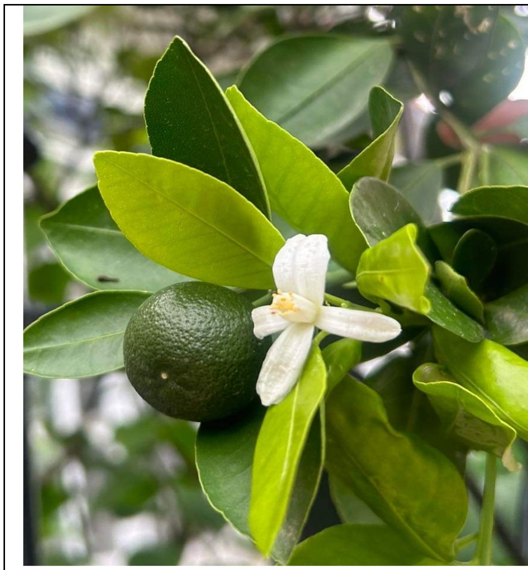

Citrus sinensis (L.) Osbeck with flowering twig

Citrus limon (L.) Osbeck with flowering twog

Plant Laboratories (NHPL), Godavari, Nepal. The lemon and orange that we had collected were identified as *Citrus limon* (L.) Osbeck and *Citrus sinensis* (L.) Osbeck. Altogether, six samples were collected, shade dried, and hydrodistilled using a clevengers' type glassware for 3 hours.
The essential oils were collected and dried over anhydrous sodium sulfate, and stored in vials at 4 ^{0}C until further analysis. In the other hand, by using the maceration method, the ethanolic extract from the powder of leaves were prepared. The supernatants were filtered with Whatman grade 1 filter paper, concentrated in rotary evaporator (Buchi), and dried extract was stored at 4 ^{0}C until further analysis (Hudz et al., 2020).

Thin layer chromatography analysis: Silica gel plate was used to analyze some major compounds and develop fingerprint of the samples. Hexane:Toluene:Ethyl acetate in the ratio of 7:3:2 was used as a mobile phase. The mixture ratio of mobile phase was optimized after the different heat and trial solvent systems. Extracts were applied to the layer of adsorbent using capillary tube and then developed into mobile phase. The developed TLC plates were placed under the UV chamber at 360 *nm* for the study (Kagan & Flythe, 2014).

GC-MS analysis of essential oils: The volatile organic compounds of the essential oils were analyzed by gas chromatography mass spectrometry (GC-MS) using Shimadzu (QP 2010 plus) with Rtx-5MS column (25m×0.25mm× 0.25μm). Total 1 μL of the essential oil diluted in acetone (1:100) was injected. The oven temperature was started at 50°C and the injection temperature was 200°C and heated up to the 250°C. The flow rate of column was maintained at 1mL/min. The qualitative analysis of the essential oil was further continued in a Shimadzu GCMS-QP2010 Plus. The detector scanning start time was 2.00 min

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and end time was 70 min. The MS library used in the analysis process was NIST 11 and FFNSC 1.3. The method was as per previously described with slight modifications (Rana et al., 2017).

RESULT AND DISCUSSION

The plant samples were collected on the basis of the wide geographical representation of Nepal. The ethanolic extracts, and essential oils were prepared from the leaves of *C. sinensis* (L.) Osbeck and *C. Limon* (L.) and phytochemically analyzed. The constituents were found variable among the samples (Table 1). The chemical composition, and their quality varied on the basis of their geographical origin (Paudel et al., 2022).

Table 1. The extractive yield of essential oils of citrus leaf samples

Sample source	Yield of extract (%)	Yield of essential oils (%)
C. limon (Koshi Province)	1.96	0.3
C. limon (Bagmati Province)	7.63	0.2
C. limon (Gandaki Province)	0.66	0.5
C. sinensis (Koshi Province)	7.12	0.2
C. sinensis (Bagmati Province)	2.78	0.3
C. sinensis (Gandaki Province)	4.38	0.4

In the present study, 45 volatile organic compounds were detected among the various essential oils samples (Table 2). Total 12, 11, and 21 compounds were identified in the lemon oil from Koshi, Gandaki, and Bagmati province respectively. Citronellal (52.77% and 27.75%) was major compound in both Koshi, and Gandaki provinces but Geranial (29.37%) was major in Bagmati province. Similarly, in the orange oil total 13, 17, and 18 compounds were identified in Koshi, Bagmati, and Gandaki province samples respectively. Both of the oils contained a higher percentage of Linalyl anthranilate (35.10% and 44.98%) as a common marker compound. The samples exhibited a chemical composition dominated by monoterpenes, and sequiterpenes which is also confirmed in previous finding (Dugo and Di Giacomo, 2002).

Based on the sample comparing with known mass spectra through characteristic fragmentation pattern, the compound was declared as Citronellal. Citronellal was observed as a dominant compound in lemon leaf but not found in the leaf of orange. Plethora of research had also confirmed

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that, limonene is the major comntituent in *C. limon* oil (Jain and Sharma, 2017, Kim et al., 2017). Linalyl anthranilate, Isopulegol, and (1R.2R.5S)-5-Methyl-2-(prop-1-en-2-yl)cyclohexanol were the common compounds detected in the lemon sample. This result is quite similar with the previous findings that some of the monoterpenes were the main constituents on lemon leaf (Hojjati and Barzegar, 2017). Similarly, α-Thujene, α-Pinene, Sabinene, β-Pinene, α-Terpine, D-Limonene, γ-Terpinene, Linalyl anthranilate, Terpinen-4-ol, and Thymol methyl ether were the common compounds detected in orange. Some researchers showed that the Indian orange leaf is rich in Eucalyptol, Sabinene, and Linalool (Periyanayagam et al., 2013). Linalyl anthranilate was the common compound detected in both lemon and orange (Meena et al., 2016). Evaluating such volatile compounds from leaf would make a valuable contribution to identification and understanding to proportion of bioactive chemical compounds of citrus leaves. Monitoring of constituents in the leaves, any manufacturers can use and maintain consistent quality and performance of their products.

Table 2. Volatile organic compounds identified in essential oil of citrus leaf samples

Compound Name	Retention Time (min)	Lemon leaf peak area percentage (Province)			Orange leaf peak area percentage (Province)		
		Koshi	Bagmati	Gandaki	Koshi	Bagmati	Gandaki
α-Thujene	12.72	-	0.97	-	4.65	5.76	3.74
α-Pinene	13.07	-	-	-	2.18	1.59	1.86
Sabinene	14.83	-	-	-	36.13	25.23	30.62
β-Pinene	15.02	-	-	-	3.29	2.22	2.6
Myrcene	15.49	-	0.6	-	3.11	1.56	2.56
α-Terpinene	16.80	-	-	-	1.44	0.82	0.47
ρ-Cymene	17.18	-	-	-	-	-	1.72
D- Limonene	17.35	-	21.05	9.52	3.67	1.74	3.07
Eucalyptol	17.54	1.11	-	-	-	-	-
β-Ocimene	18.18	2.58	-	-	7.07	-	6.83
trans-β-Ocimene	18.18	0.82	0.48	-	-	-	-
1,3,6-(Z)-Octatriene,3,7-dimethyl	18.21	-	1.53	-	-	4.02	-
Melon aldehyde	18.49	0.66	-	-	-	-	-
γ-Terpinene	18.81	-	-	-	4.12	2.65	2.17
Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl	19.25	-	-	-	-	2.08	1.59
Cyclohexene, 1-methyl-4-(1-methylethylidene)	20.25	-	-	-	1.66	-	-
2-Carene	20.26	-	-	-	-	-	0.51
Linalyl anthranilate	20.69	20.08	1.68	8.73	25.88	44.98	35.1
Benzene, 1 methyl-3-(1-methylethenyl)-	20.75	-	-	-	-	-	0.53

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Trans-Rose oxide	21.27	0.53	-	-	-	-	-
Isopulegol	23.05	2.48	0.49	2.03	-	-	-
Citronellal	23.27	52.77	1.32	27.75	-	-	-
(1R,2R,5S)-5-Methyl-2-(Prop-1-en-2-yl)cyclohexanol	23.59	4.19	1.06	3.92	-	-	-
Isoneral	23.80	-	0.52	-	-	-	-
Terpinen-4-ol	24.60	-	-	-	2.75	1.61	1.4
Isogeranial	24.65	-	3.1	0.84	-	-	-
α-Terpineol	25.24	-	-	-	-	0.77	0.7
n-Decanal	25.72	-	0.8	-	-	-	-
Citronellol	26.80	11.23	-	14.2	-	-	-
Thymol methyl ester	27.18	-	-	-	2.92	1.87	3.17
Neral	27.49	-	24.95	11.79	-	-	-
Nerol	28.06	-	1.48	4.6	-	-	-
Geranial	28.83	-	29.37	14	-	-	-
Citronellyl acetate	32.41	2.91	-	-	-	-	-
2,6-(Z)-Octadien-1-ol, 3,7-dimethyl-, acetate	32.93	-	1.58	-	-	-	-
Geranyl acetate	33.76	-	4.3	-	-	-	-
E-Geranyl acetate	33.76	0.64	-	2.62	-	-	-
E-Caryophyllene	35.72	-	1.99	-	-	-	-
β-Selinene	38.53	-	0.64	-	-	-	-
(1S,2E,6E,10R)-3,7,11,11-Tetraethylbicyclo	38.96	-	-	-	-	1.37	-
(E,E)-, α -Farnesene	39.02	-	1.37	-	-	-	-
E- β-Famesene	39.02	-	-	-	-	0.82	-
β-Bisabolene	39.17	-	0.72	-	-	-	-
β-Sinensal	46.51	-	-	-	1.14	0.91	-
2,6,11-Dodecatruenal, 2,6-dimethyl-10-methylene	46.52	-	-	-	-	-	0.71

GC-MS chromatogram of essential oils of *Citrus limon*(L.) Osbeck and *Citrus sinensis*(L.) Osbeck. are provided the appendix section.

The presence of citronellal at such high concentration shows that the enzyme responsible for its synthesis is suppressed, resulting in the absence of intermediate intermediates (Dewick, 2009). Geranial and neral, which acts as intermediary products in the biosynthetic pathways, are abundant in *Citrus limon* from Bagmati province. Although the ultimate product, citronellal, is present, its concentration is low. This indicates that the biosynthesis process is still in progress and that the high amounts of intermediate products have not been completely transformed into the end product. Consequently, the smaller amount of the end product is due to the ongoing conversion of

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intermediates. This observation supports that the chemical composition can vary across geographical regions, altering biosynthetic pathways and resulting and varying amounts of intermediate and end products (Kazachkova et al., 2024). The compounds present in *Citrus sinensis (L.)* Osbeck leaves were analyzed in relation to altitude. Increasing the altitude found increased the percentage of linalyl anthranilate but the percentage of D-Limonene and Sabinene were decreased. The biosynthesis of terpenes in altitudes is influenced by light, temperature, soil moisture, and vegetation of that area. Monoterpenes metabolites are markedly contributing to stress tolerence, particularly at high altitudes (Večeřová et al., 2021). The compounds of *Citrus limon (L.) Osbeck* leaf were found that as the altitude increases, the percentage of linalyl anthranilate is decreases. This result is slightly different with the previous finding that the altitude affected the biosynthesis of oxygenated monoterpenes generally and were greatest when low; while sesquiterpene constituents were greatest at high altitudes (Şanli and Karadoğan, 2016).

TLC fingerprint was remarkable on composition variation among samples (appendix). After several testing, we optimized the mobile phase as 7:3:2 mixture of hexane, toluene, and ethyl acetate. TLC plate at 360 nm UV light showed the variation in metabolite composition. This TLC-based screening method can be used in the preparation of chemical fingerprint of botanical material as well as analytical aspect related to quality control, which is also described previously (Kowalska and Sajewicz, 2022). This evidence can be applied to answer the number of components in the mixture, and quality control of these essential oils.

CONCLUSION

The comparative analysis on the phytochemical profile of alcoholic extract, and essential of *Citrus limon* (L.) Osbeck and *Citrus sinensis* (L.) Osbeck leaves from Nepal showed the variations in their compositions. Linalyl anthranilate, which was confirmed by its mass spectra, was a common compound distributed in all leaf samples. The concentration of Linalyl anthranilate was found decreased with increasing the altitude. Similarly identified other compounds such as Citronellal, geranial, neral, D-limonene, were dominant in lemon leaves, where as Linalyl anthranilate, Sabinene, α-Thujune, β-Ocimine were dominant in orange leaves. Mobile phase 7:3:2 mixture of hexane, toluene, and ethyl acetate was optimized for TLC, which can be used for the quality control of leaf extracts. The present study helps to revel the constituents of the both essential oils but also helpful in searching for bioactive agents from specific location of Nepal. Linalyl anthranilate compound could be a compound of chemotaxonomic study for further study of citrus family.

DECLARATION OF CONFLICT OF INTEREST

No conflict of interest to declare.

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DECLARATION OF HONOUR

We declare on our honour that our results are not fake and made up.

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APPENDIX

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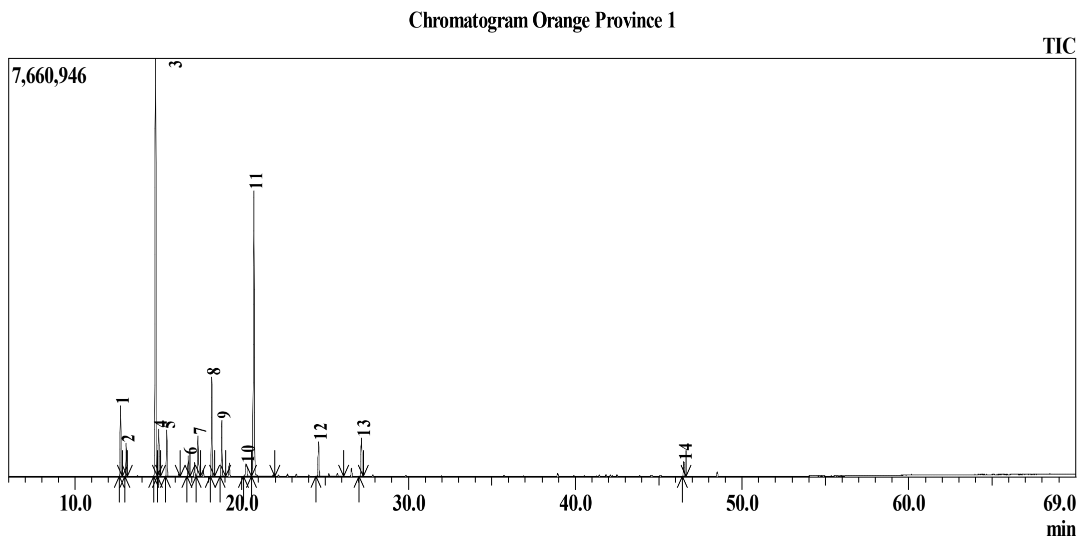
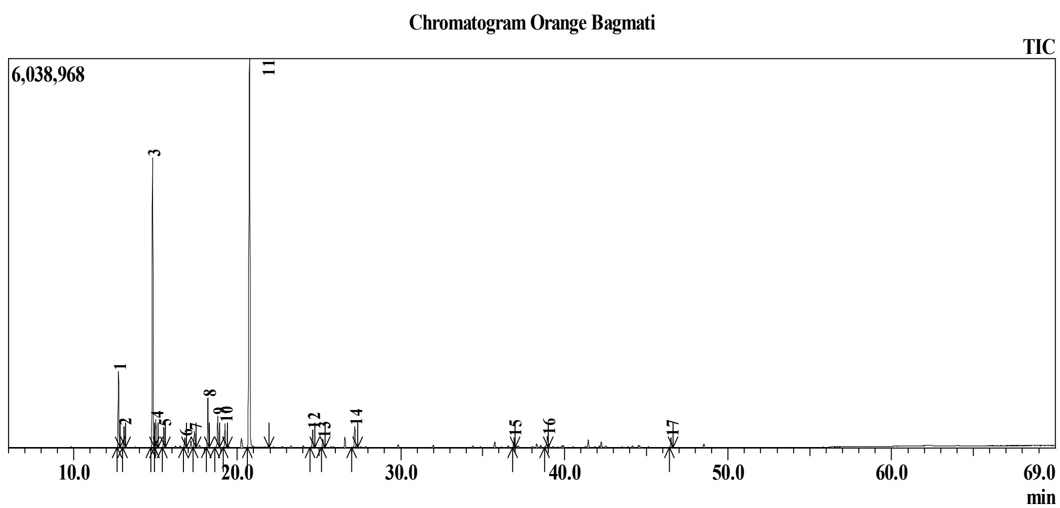
GC-MS chromatogram of essential oils of *Citrus limon*(L.) Osbeck and *Citrus sinensis*(L.) Osbeck.





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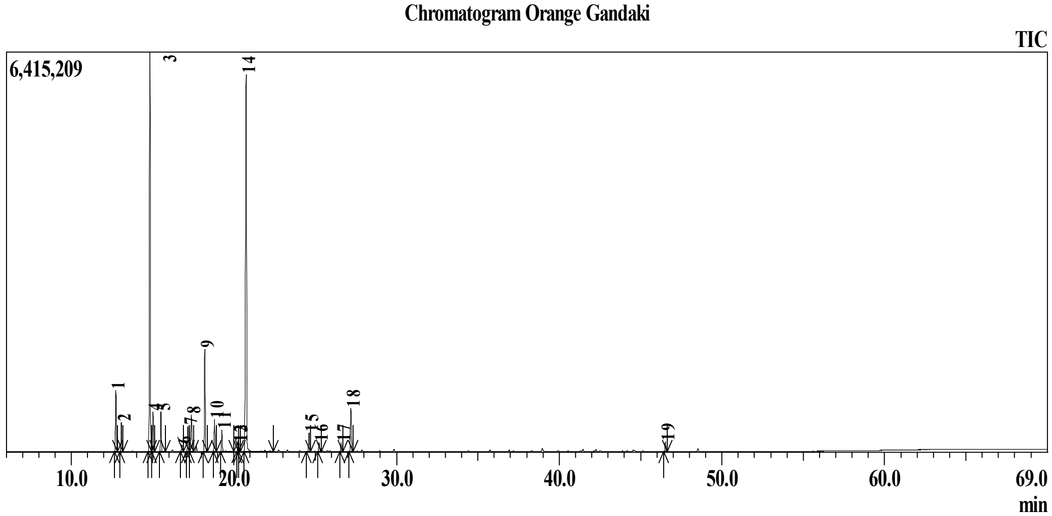
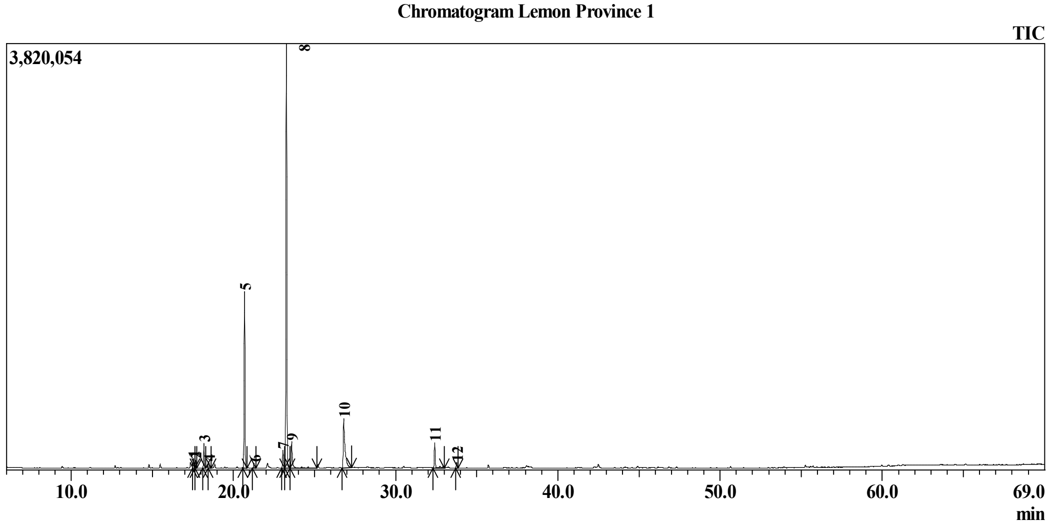
Chromatogram Orange Gandaki



Chromatogram Lemon Province 1



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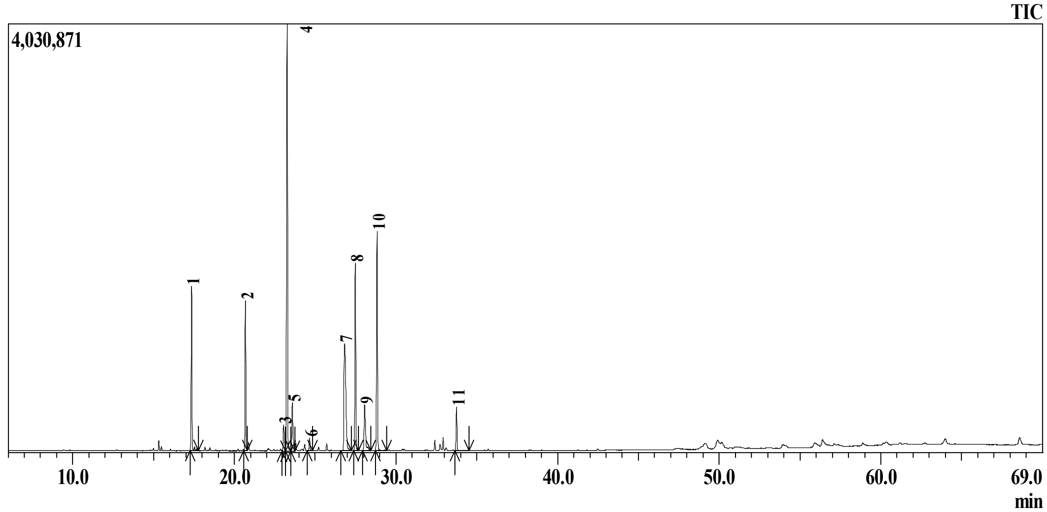
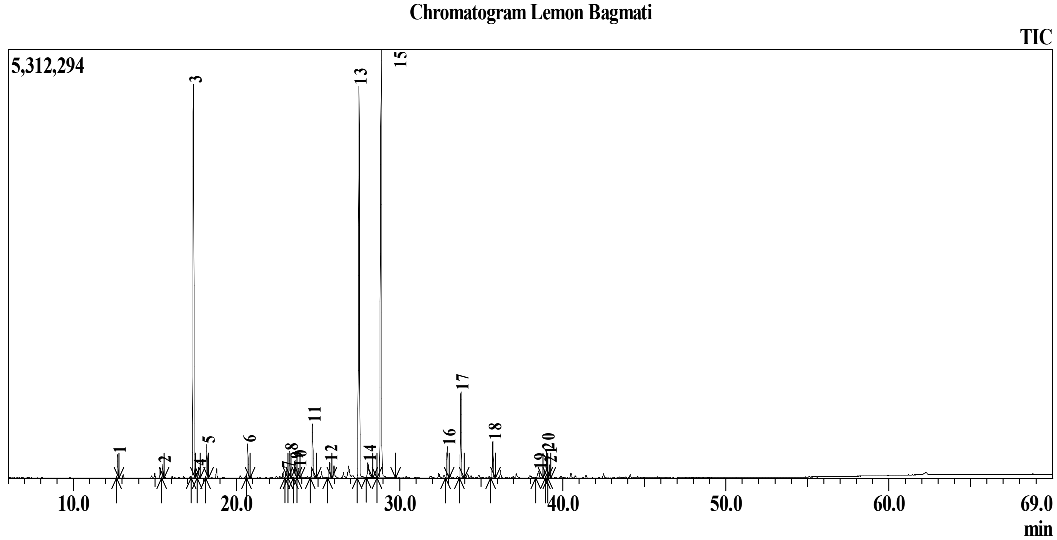
Chromatogram Lemon Gandaki



Chromatogram Lemon Bagmati



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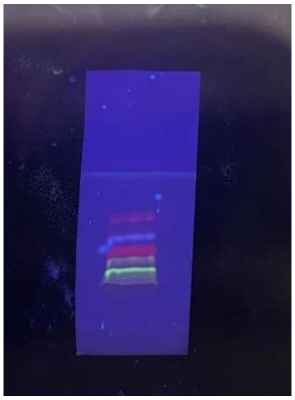
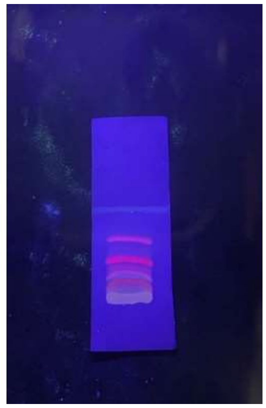
TLC of orange and lemon leaf extracts from different provinces of Nepal


Koshi Province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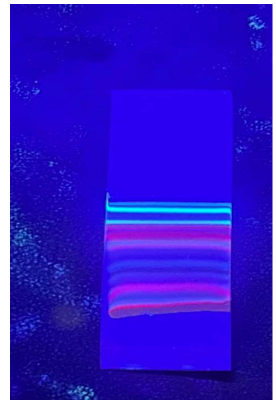
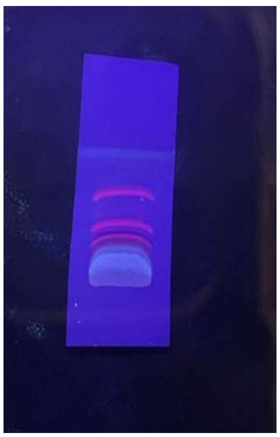
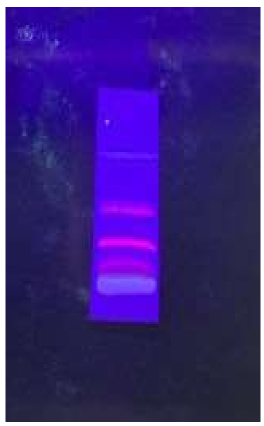
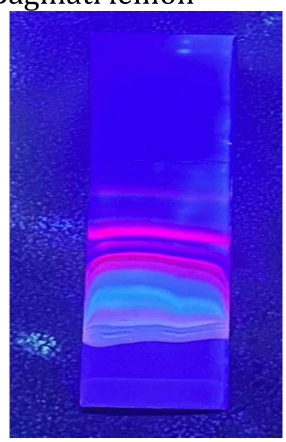

Bagmati lemon


Koshi Province orange


Gandaki Orange


Bagmati orange

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