CERTIFICATE

This is to certify that the final report on UGC Minor research project entitled **"Study of chemical compositions of the fixed and essential oils of *Anisomeles malabarica* R.Br with anti-arthritic activity and its comparison with commercially available oil using analytical methods."** is a record of bonafide research work carried out by Dr. Manish S Hate, Associate Professor of Chemistry, Ramnarain Ruia Autonomous College, Matunga, Mumbai, Maharashtra.

A copy of the final report of Minor Research Project has been kept in the library of Chemistry Department and an executive summary of the report has been posted on the website of the College.

Principal
I/c Principal
Ramnarain Ruia Autonomous College
Matunga, Mumbai - 400 019.

College Seal

RAMNARAIN RUIA COLLEGE 1937 MUMBAI-19

Ref. No.:

Date:

To,
The Joint Secretary,
University Grant Commission,
WRO, Ganeshkhind, Pune – 411007

Subject: Submission of the Final Report of Minor Research Project with PCR.
Reference: Approval Letter No.: UGC Ref. No. F.47-1201/14 (SC/34/WRO)

Sir/Madam,

With reference to the above mentioned subject, I am submitting herewith the Project Completion Report of Minor Research Project sanctioned to Dr. Manish S Hate entitled **"Study of chemical compositions of the fixed and essential oils of *Anisomeles malabarica* R.Br with anti-arthritic activity and its comparison with commercially available oil using analytical methods"** along with required statements, Certificates, Reports and PCR. I request you to accept the same and release the remaining amount of the project.

Thank You,

Yours Faithfully,

Enclosed;

1. UGC MRP Award Letter dated 16/08/2014
2. Acceptance Certificate
3. UGC Amount Release Letter dated 16/03/2017
4. Audited Consolidated Utilisation Certificate
5. Consolidated Statement of Expenditure
6. Statement of Expenditure on Field Work
7. Final Report of the work done
8. Assets Certificate
9. Accession Certificate
10. Request Letter for Reimbursement of Rs. 85,000/- as second instalment.
11. Date of Starting 15/04/2017 and date of Completion of the project 02/04/2019
12. A copy of summary of report uploading on the college website.

RAMNARAIN RUIA COLLEGE 1937 MUMBAI-19

Principal,

ज्ञान-विज्ञान विमुक्तये

विश्वविद्यालय अनुदान आयोग
University Grants Commission
मानव संसाधन विकास मंत्रालय, भारत सरकार
Ministry of Human Resource Development, Govt. of India
पश्चिम विभागीय कार्यालय गणेशखिंड,पुणे- ४११००७
Western Regional Office, Ganeshkhind, Pune – 411007

Ph: 020 – 25696896, 25696897
Tele.Fax:.- (020-25691477
Website- www.ugc.ac.in
Email: mrpugcwro@gmail.com

RUIA COLLEGE Inward No. 10 Dated 3.4.17

SPD 03/04/17

No. F. 47-1201/14 (SC/ 34/ WRO) XII Plan

Date: 16 MAR 2017

The Drawing and Disbursing Officer,
University Grants Commission,
Pune – 411007.

UGC Minor Res Proj Dr Manish Hate

Subject: RELEASE OF GRANTS TO RAMNARAIN RUIA COLLEGE, L.N. ROAD,, CENTRAL MATUNGA, MUMBAI, PIN - 400019 FOR THE YEAR 2016-2017 UNDER PLAN FINANCIAL ASSISTANCE TO TEACHER IN COLLEGES FOR UNDERTAKING MINOR RESEARCH PROJECTS – RELEASE OF FIRST INSTALLMENT.

Sir/Madam,

I am directed to convey the sanction of the Commission. The UGC on the recommendations of the Expert Committee has approved the Minor Research Project in the subject of **Chemistry** entitled "Study of chemical compositions of the fixed and essential oils of Anisomeles malabarica (Linn.) R. Br. Ex Sims with anti-arthritic activity and its comparison with commercially available oil using analytical methods" to be undertaken by **Dr. Hate Manish,** of **RAMNARAIN RUIA COLLEGE, L.N. ROAD,, CENTRAL MATUNGA, MUMBAI-400019.** The financial assistance of the UGC would be limited to Rs. 155000/- (Rupees One Lakh FiftyFive Thousand only) for a period of two years. An amount of Rs. 85000/- (Rupees EightyFive Thousand only) is presently being sanctioned as the first installment Plan expenditure to be incurred during 2016-2017.

(In Rupees)

Non-Recurring Grant for Two years	Amount (Rs)	Recurring grant	1st Year Amount	2nd Year Amount	(Comp- SC) Grant to be approved as Ist Inst.
Books & Journals	15000	Contingency	25000	25000	NR 100% Rec. 1st Year
Equipment	0	Special Need	0	0	
		Travel/Field work	5000	5000	
		Chemicals & Glassware	40000	40000	
		Others	0	0	
Total (Rs.)	15000		70000	70000	85000

Total amount for the project: Rs. 155000/-

NOTE:

- **For remittance of refund to UGC (WRO), Bank details may be seen at point – 9.**
- **The grants should be utilized within the time period as specified under the GFR, 2005.**
- **"The University/College/Institution is registered/ mapped with PFMS portal" and settled.**

1. **The sanctioned amount is debitable to the Plan Head 3 (31) and is valid for payment during the financial year 2016-17 only.**

Component	Head of A/c	General –In-aid (31)	Capital Assets (35)	Total
SC	3(B)	70000	15000	85000/-

2. The amount of the grants shall be drawn by the Drawing & Disbursing Officer, UGC (WRO), Pune on the Grants-in-aid bill and shall be disbursed to and credited to the Principal of the college through Electronic mode as per the following details:

a.	Details (Name & Address) of Accounts Holder:	RAMNARAIN RUIA COLLEGE, L.N. ROAD,, CENTRAL MATUNGA, MUMBAI, PIN- 400019
b.	Account No.:	623701125549
c.	Name & address of Bank Branch	ICICI, MATUNGA BRANCH
d.	MICR Code:	
e.	IFSC Code:	ICIC0006237
f	Type of Account:	Saving Account

3. The grant is subject to adjustment on the basis of Utilization Certificate in the prescribed Performa submitted by the University/ College/ Institution.
4. The University/ College shall maintain proper accounts of the expenditure out of the grants, which shall be utilized, only on approved items of expenditure.
5. The University/ Institution may follow the General Financial Rules, 2005 and take urgent necessary action to amend the manuals of financial procedures to bring them in conformity with GFRs, 2005 and those don't have their own approved manual on financial procedures may adopt the provision of GFRs, 2005 and instruction/ guideline there under from time to time.
6. The Utilization Certificate to the effect that the grant has been utilized for the purpose for which it has been sanctioned shall be furnished to UGC as early as possible after the close of current financial year.

7. The assets acquired wholly or substantially out of UGC's grant, shall not be disposed of or encumbered or utilized for purposes other than those for which the grant was given, without proper sanction of the UGC, and should at any time the College cease to function, such assets shall revert to the University Grants Commission.
8. A Register of the assets acquired wholly or substantially out of the grant shall be maintained by the University/ College in the prescribed proforma.
9. The grantee institution shall ensure the utilization of grants-in-aid for which it is being sanctioned/ paid. In case non-utilization / part utilization, the simple interest @ 10% per annum as amended from time to time on unutilized amount from the date of drawl to the date of refund as per provision contained in General Financial Rules of Govt. of India will be charged.

In case of unspent balance/refund may be remitted to UGC (WRO) through RTGS as per the following bank details.

Account Name	Name of Bank and branch name	IFSC Code	Component	Bank Account No.
Joint Secretary UGC(WRO), Pune	Canara Bank Model Colony, Pune	CNRB0000262	SC	262101060500

10. Interest earned by the College/Institution against UGC grants, if any, will be treated as an additional grant and must be specifically incorporated in the statement of expenditure while submitting it to UGC (WRO).
11. The Univ./College shall follow strictly the Government of India/ UGC's guidelines regarding implementation of the reservation policy [both vertical (for SC, ST & OBC) and horizontal [for persons with disability etc.)] in teaching and non-teaching posts.
12. The University/ College shall fully implement to Official Language Policy of Union Govt. and comply with the Official Language Act, 1963 and Official Languages (Use for Official Purposes of the Union) Rules, 1976 etc.
13. The sanction issues in exercise of the delegation of powers vide UGC office order No. 69/2014 [F. No. 10-11/12 (Adm IA & B)] dated 26/3/2014.
14. **"The University/ Institution shall strictly follow the UGC Regulations on curbing the menace of Ragging in Higher Education Institutions, 2009" and amendments thereof.**
15. The University/ Institution shall take immediate action for its accreditation by National Assessment & Accreditation Council (NAAC). NBA/authorised accreditation body.
16. The accounts of the University/ Institution will be open for audit by the Comptroller & Auditor General of India in accordance with the provisions of General Financial Rules, 2005.
17. The annual accounts i.e. balance sheet, income and expenditure statement and statement of receipts and payments are to be prepared strictly in accordance with the Uniform Format of Accounting prescribed by Government.
18. An amount of Rs._____ /- out of Rs. _____/- has been utilized against this office sanction letter of even dated____ by the university/Institute/College vide Ref No. ____ dated for the purpose for which it was sanctioned and noted in grant in aid/ BCR register at Pg. No. 1 & S. No.____
19. Future grant will be released on receipt of Statement of Expenditure Utilization Certificate (Item-wise).
20. Funds to the extent are available under the Scheme.
21. This issues with the concurrence of IFD vide Diary No. ____(IFD) dated___ (N. A.)
22. This issues with the approval of Head of Office.
23. Entry has been made in BCR at Pg. No. 1 & S. No. 34.

Yours faithfully

(Dr. G. Srinivas)
Joint Secretary

Copies forwarded for information and necessary action to:

1. **THE PRINCIPAL**
   RAMNARAIN RUIA COLLEGE, L.N. ROAD,, CENTRAL MATUNGA, MUMBAI, PIN-400019.
2. **DR. HATE MANISH, RAMNARAIN RUIA COLLEGE, L.N. ROAD,, CENTRAL MATUNGA, MUMBAI, PIN - 400019.**
3. **DIRECTOR (BCUD), MUMBAI UNIVERSITY, MG ROAD, FORT, MUMBAI, PIN- 400032**
4. **DIRECTOR, HIGHER EDUCATION, CENTRAL BUILDING, NEAR PUNE RAILWAY STATION, PUNE, PIN- 411001**
5. **ACCOUNTANT GENERAL, MAHARASHTRA STATE, 101, MAHARSHI KARVE MARG, MUMBAI- 400020**
6. **GUARD FILE.**

(Dr. R. Manoj Kumar)
Deputy Secretary

UNIVERSITY GRANT COMMISSION
WESTERN REGIONAL OFFICE
GANESHKHIND, PUNE – 411007

ACCEPTANCE CERTIFICATE FOR RESEARCH PROJECT

Name: Dr. Manish Shamrao Hate

No. F. 47-1201/14 dated 16th March 2017

Title of the project: "Study of chemical compositions of the fixed and essential oils of *Anisomeles malabarica* R.Br with anti-arthritic activity and its comparison with commercially available oil using analytical methods."

1. The research project is not being supported by any other funding agency.
2. The terms and conditions related to the grant are acceptable to the Principal Investigator and College/~~Institute~~.
3. At present, I have no research project approved by UGC and the account for the previous project, if any have been settled.
4. The College/ ~~Institute~~ is fit to receive financial assistance from UGC and is included in the list of Section 2(f) & 12(B) prepared by the UGC. **The College is not self – financing.**
5. The Principal Investigator is a retired teacher and eligible to receive honorarium as he/~~she~~ is neither getting any honorarium from any agency nor is he/~~she~~ gainfully employed anywhere.
6. (i) His / ~~Her~~ date of birth is 26th August 1969
   (ii) Age: 48
7. The date of implementation of the project is **16th March 2017**
8. The Principal Investigator is a permanent/ ~~regular~~ teacher.
9. The Principal Investigator is undertaking the above project after a gap of year from the last UGC project completed satisfactorily.

Principal Investigator

This is also to certify that the college is self financing and is charging fees as per the State/ University norms.

Principal
College: Ramnarain Ruia College
(Seal)
Date: 15/04/2017

(Please strike out whichever is not applicable)

UGC(WRO), Pune

Documents Required for Settlement of Minor Research Projects

STATEMENT OF EXPENDITURE

Name of Principal Investigator: Dr. Manish S Hate

Name of the College: Ramnarain Ruia Autonomous College

Date of Starting of project **02/04/2016** and date of completion of Project **31/03/2019**.

Head	Sanctioned Amount	Received Amount	Actual Expenditure
Books and Journals	15,000/-	15,000/-	15,018/-
Equipment	0	0	0
Contingencies	50,000/-	50,000/-	50,004/-
Special Need	-	-	-
Travels	10,000/-	10,000/-	10,266.60/-
Chemicals	80,000/-	80,000/-	84,811/-
Others	-	-	-
Total	**1,55,000/-**	**1,55,000/-**	**1,60,099.60/-**

Signature of the Principal Accountant with seal

Signature of the Principal Investigator

28/05/19

Signature of the Chartered with Seal and Regd. No. of C.A.

UDIN: 19105525AAAAD05897

UGC(WRO),Pune

Documents Required for Settlement of Minor Research Projects

UTILIZATION CERTIFICATE FOR TOTAL/UTILIZED GRANT

It is certified that the grant of Rs.1, 55,000/-(Rupees One Lakh Fifty Five Thousand only) sanctioned to Dr. Manish S Hate by University Grants Commission vide their letter No. 47-1201/14(SC/34/WRO) XII Plan dated 16 March 2017 towards Minor Research Project has been fully utilized for the purpose for which it has been sanctioned and in accordance with the terms and conditions laid down by the commission.

If as a result of check or audit objection, some irregularity is noticed at a later stage action will be taken to refund or regularize the objected amount.

Total actual expenditure incurred for this project is of Rs. **1, 60,099.60/-** Rupees (One Lakh Sixty Thousand Ninety Nine Rupees and Sixty Paisa only)

Signature of the Principal	Signature of the Principal	Signature of the Chartered
~~Accountant~~ with Seal	Investigator	with Seal and Regd. No. of C.A.
		28/05/19
		UDIN: 19105525AAAAD05897

The Institute of Chartered Accountants of India
(Set up by an Act of Parliament)

Unique Document Identification Number(UDIN) for Practicing Chartered Accountants

DOCUMENT DETAILS

Status Date/Time:	28-05-2019 08:47:43
Unique Document Identification Number (UDIN):	19105525AAAADO5897
Member Details:	ANAGHA NITIN THATTE (105525)
Document Type:	Certificates
Type of Certificate:	Others
Date of signing of Document:	28-05-2019
Figures/Particulars:	1. Sanction Amount: 1,55,000.00 2. Received Amount: 1,55,000.00 3. Actual Expenditure: 1,60,099.60
Document Description:	UGC Grant for Minor Research - Dr. Manish S Hate
Status:	Active

VERIFY ANOTHER UDIN| EXIT

CHITALE MUMBAI

DISCLAIMER

This UDIN System has been developed by ICAI to facilitate its members for verification and certification of the documents and for securing documents and authenticity thereof by Regulators.

However, ICAI assumes no responsibility of verification and certification of document(s) carried out by the Members and the concerned member(s) shall alone be responsible therefore.

UNIVERSITY GRANT COMMISSION
WESTERN REGIONAL OFFICE
GANESHKHIND, PUNE – 411007

PERFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WROK DONE ON THE PROJECT

1) **TITLE OF THE PROJECT:** "Study of chemical compositions of the fixed and essential oils of *Anisomeles malabarica* R.Br with anti-arthritic activity and its comparison with commercially available oil using analytical methods."

2) **NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR:**
Manish Hate, A/305, Highland Ocean CHS, Charkop Village, Kandivali (west), Mumbai-400067. manish.manish.hate@gmail.com / 09987516998

3) **NAME AND ADDRESS OF THE INSTITUTION:**
Ramnarain Ruia Autonomous College, L. N. Road, Matunga, Mumbai -400019

4) **UGC APPROVAL NO. AND DATE:** 47-1201/14 dated 16th March 2017

5) **DATE OF IMPLEMENTATION:** 15/04/2017

6) **TENURE OF THE PROJECT: Two Years (2017-2019)**

7) **TOTAL GRANT ALLOCATED:** 155000/-

8) **TOTAL GRANT RECEIVED:** 85000/-

9) **FINAL EXPENDITURE:** 160099.60/-

10) **OBJECTIVES OF THE PROJECT:**

- To standardize plant extracts using different analytical methods like Gas Chromatography-Mass Spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC) and High Performance Thin Layer Chromatography (HPTLC).
- To compare the fixed and essential oils extracted from the selected plant with commercially available oil.

- To evaluate anti-arthritic activity of *Anisomeles malabarica* (Linn.) R. Br. ex Sims.

11) WHETHER OBJECTIVES WERE ACHIEVED (GIVE DETAILS): Yes

In the present research project following objectives were achieved in two phases;

Phase I

- Detailed literature survey were carried out
- *Anisomeles malabarica* (Linn.) R. Br.ex Sims plant was collected from the Dindigul district of Tamil Nadu.
- Herbarium was prepared and authenticated at Botanical Survey of India, Pune.
- Proximate analyses were performed and fixed and Essential oils were extracted using various extraction techniques.

Phase II

- Gas Chromatography-Mass Spectrometry (GC-MS) was developed for profiling of Fixed and essential oils and High Performance Thin Layer Chromatography (HPTLC) method were developed and validated from *Anisomeles malabarica* (Linn.) R. Br.ex Sims plant.
- Fixed and essential oils extracted from the selected plant were compared with commercially available oil.
- Finally Anti-arthritic activity of *Anisomeles malabarica* (Linn.) R. Br. ex Sims were evaluated using In – Vitro model.

12) ACHIEVEMENTS FROM THE PROJECT:

Phytochemical screening of the aerial parts of the plants of *Anisolmeles malabarica* revealed the presence of flavanoids and these natural products are responsible for anti-inflammatory and antioxidant activity.

Thus, the results of this study confirmed the traditional uses, claiming that the plant *Anisolmeles malabarica* have potent anti-rheumatic, anti-inflammatory and anti-oxidant properties.

The developed and validated Chromatographic methods can be used for qualitative and quantitative routine analysis.

13) SUMMARY OF THE FINDINGS (IN 500 WORDS):

In physicochemical analysis of *Anisomeles malabarica* R.BR, the highest extractive value was obtained from water and Methanol. High alcohol soluble and water soluble extractive values reveal the presence of polar substance like phenols; tannins and glycosides.

In qualitative phytochemical analysis, phenolics, flavonoids, and alkaloids class of compounds were present in high amount as compared to other phytoconstituents analyzed. Hence, the determination of physico and phytochemical profile of

Anisomeles malabarica R.BR may be useful to supplement information in respect to its identification, authentication and standardization of herbal drugs.

During extraction of essential oil two techniques Steam distillation and Hydro-distillation were employed. The investigation of the effect of percent yield obtained from these techniques shows that Hydro-distillation method is superior over steam distillation.

For fixed oil extraction hot continuous extraction method i.e. Soxhlet Extraction method were used. The percentage amount yield of fixed oils present in the leaves shows high percent content of fixed oil in the selected plant *Anisomeles malabarica* R.BR.
Physicochemical analysis of essential and fixed oil extracted from *Anisomeles malabarica* R.BR shows great variation. The essential oils of *Anisomeles malabarica* R.BR was found to be pale yellow in colour and fixed oil was colourless. Both the volatile had pleasant odour while fixed oil had unpleasant odour.

Fixed oils have high specific gravity values as compared to essential oils of both plants. The optical rotation was found to be high in fixed oils of both the plants. Saponification values determined and found to be very high in *Anisomeles malabarica* R.BR. All the oil samples are found in the range reported for plants (lower than the 188-196) but not useful in soup industry as these values are much lesser than the required value (±300).

The iodine valve observed for the oil samples in the present study were between 100-160g/100g oil. These values were found in the permissible range for semi-drying of oil (100-300). Recorded data for iodine number showed that all the oil samples have high unsaturated fatty acids contents.

In Chromatographic analysis, Gas Chromatography Mass Spectrometry methods were developed for the extracted essential and fixed oils from the selected plant *Anisomeles malabarica* R.BR. The developed GCMS method can be used for the identification of essential and fixed oil constituents from these plants as a quality parameter.
The present research work has been concerned with determining and comparing the chemical compositions of essential oil distilled from the *Anisomeles malabarica* R.BR plants. The chemical analysis by GCMS have allowed us to identified total 38 essential oil components from the *Anisomeles malabarica* R.BR leaves and flowers. This shows that the *Anisomeles malabarica* R.BR plants have more essential oil components.

The fixed oils detected by GCMS were high in *Anisomeles malabarica* R.BR flowers. The biological activities associated with the constituents identified by GCMS were compared by Dr. Duke's database and Pubmed data available from the previously isolated components of different plants.

Simultaneous HPTLC method were developed and validated for Gallic acid and Quercetin in *Anisomeles malabarica* R.BR. The developed HPTLC methods are simple, accurate and precise and can be used for regular quality control analysis of aerial powders of these plants.

Biological activities of different extracts of both the plant were evaluated using In-vitro model: DPPH assay and Heat Induced Hemolysis.

14) CONTRIBUTION TO THE SOCIETY (GIVE DETAILS):

Simultaneous HPTLC method can be used for regular quality control analysis of aerial powders of *Anisomeles malabarica* R.BR plant.

These studies have shown that the methanol extract of *Anisomeles malabarica* R.BR contain some active ingredients with the potential of being good anti-inflammatory and antioxidant agents. NSAIDs like diclofenac, used as standard drug in anti-inflammatory study, is having good anti-inflammatory and analgesic property, but is also having side effects on liver. Therefore *Anisomeles malabarica* R.BR may become the alternative to the NSAIDs. For that, further study for detailed investigation of the mechanism of action is needed.

15) WHETHER ANY PH.D ENROLLED/ PRODUCED OUT OF THE PROJECT:

A student was registered for PhD earlier before the sanctioning of MRP. The PhD topic entitled "Comparative studies of essential oil compositions of Anisomeles *malabarica* (Linn.) R. Br. ex Sims and Semecarpus *anacardium* Linn. f. plants with Anti arthritic activity and its comparison with commercially available oils using analytical methods" at Department of Chemistry, Ramnarain Ruia Autonomous College, University of Mumbai.

16) NO. OF PUBLICATIONS OUT OF THE PROJECT (PLEASE ATTACH REPRINTS):

SIGNATURE OF THE PRINCIPAL INVESTIGATOR

RAMNARAIN RUIA COLLEGE 1937 MUMBAI-19

PRINCIPAL

(SEAL)

UNIVERSITY GRANT COMMISSION

STATEMENT OF EXPENDITURE INCURRED ON FIELD WORK

Name of the Principal Investigator: Dr.Manish S.Hate

Sr. No.	Name of the place visited	Duration of the visit	Mode of Journey	Expenditure Incurred (Rs.)
01	Mumbai to Solapur(Field work)	28/03/2017	Train	995 /-
02	Solapur to Pune(Plant collection)	30/05/2017	Train	125 /-
03	Mumbai to Umargam (plant collection)	27/04/2017	Train	305 /-
04	Ruia college to Wagle estate (Field visit)	21/12/2017	UBER taxi	425.60 /-
05	Borivali to Ruia college	13/12/2017	OLA taxi	497 /-
06	Ruia college to Malad	13/12/2017	OLA taxi	480 /-
07	CSTM to Innani college,Amravati	22/03/2018	Train	2060 /-
08	CSTM to Innani college,Amravati	22/03/2018	Train	2060 /-
09	Amravati Railway Station to Innani college,karanja	22/03/2018	Bus	154 /-
10	Karanja to Amravati Railway Station	23/03/2018	Taxi	825 /-
			TOTAL (2017-2018)	**7926.60 /-**
11	Airoli to J.S.M College,Alibag	08/03/2019	OLA taxi	2340 /-
			TOTAL (2018-2019)	**2340 /-**
			GRAND TOTAL	**10266.60/-**

Certified that the above expenditure is in accordance with the UGC norms for Minor Research Projects

SIGNATURE OF PRINCIPAL INVESTIGATOR

RAMNARAIN RUIA COLLEGE 1937 MUMBAI-19

Principal
Ramnarain Ruia College
Matunga, Mumbai - 400 019

PRINCIPAL

(Seal)

UGC(WRO), Pune

Documents Required for Settlement of Minor Research Projects

ACCESSION CERITIFICATE

It is certified that the Books purchased form Capital Grant are handed over to the college central/ departmental library. Their Accession Number is from **134124 to 134135, 134719 to 134724, 134764 to 134769 and 134830 to 134831.**

Signature of the Principal
Accountant with seal

RAMNARAIN RUIA COLLEGE 1937 MUMBAI-19

Signature of the Principal Investigator

UGC(WRO), Pune

Documents Required for Settlement of Minor Research Projects

ASSESTS CERITIFICATE

It is certified that the following Equipment has been handed over to the college.

1) _____
2) _____
3) _____
4) _____

NA

Signature of the Principal
~~Accountant~~ with seal

Signature of the Principal Investigator

MINOR RESEARCH PROJECT

Title

"Study of chemical compositions of the fixed and essential oils of *Anisomeles malabarica* R.Br with anti-arthritic activity and its comparison with commercially available oil using analytical methods."

Report submitted

To

The joint secretary and Head
Western Regional Office (WRO)
University grants commission
Ganeshkind
Pune - 411007

During XII Plan period
2016-2018

By

Principal Investigator
Dr. Manish S Hate
Associate Professor of Chemistry
Department of Chemistry
Ramnarain Ruia Autonomous College
Matunga, Mumbai-400019
Maharashtra

June 2019

CERTIFICATE

This is to certify that the final report on UGC Minor research project entitled **"Study of chemical compositions of the fixed and essential oils of *Anisomeles malabarica* R.Br with anti-arthritic activity and its comparison with commercially available oil using analytical methods."** is a record of bonafide research work carried out by Dr. Manish S Hate, Associate Professor of Chemistry, Ramnarain Ruia Autonomous College, Matunga, Mumbai, Maharashtra.

A copy of the final report of Minor Research Project has been kept in the library of Chemistry Department and an executive summary of the report has been posted on the website of the College.

Principal
I/c Principal
Ramnarain Ruia Autonomous College
Matunga, Mumbai - 400 019.

College Seal

RAMNARAIN RUIA COLLEGE 1937 MUMBAI-19

Acknowledgement

I express the deep sense of gratitude to the Joint Secretary and Head WRO, UGC, Pune, for sanctioning the minor research project under XII Plan period.

I acknowledge with gratitude for the co-operation and guidance extended to me by Vice Chancellor, Mumbai University, and Former Principal Prof. Suhas Pednekar.

I am grateful to the Principal Dr. Anushree Lokur for the full co-operation and guidance extended.

I have great pleasure in expressing my heartfelt thanks to both the Vice Principals of the college, Dr. Sucheta Ketkar and Dr. Vaibhavi Palsule.

My special thanks to Head of Chemistry, Dr. Jyoti S Patwardhan and Dean Research Dr. Sunita Shailajan for providing facilities, to carry out research work.

I extend my respectful thanks to the College Management S P Mandali for their support and co- operation.

I take the opportunity to thank my Colleagues Dr. Mohmad Vasim Sheikh and Ms. Dipika Darekar.

INDEX

CHAPTER I

SECTION I

INTRODUCTION

INTRODUCTION:

From the evolution, human being daily come across the various plant parts like fruits, flowers, leaves, stems, barks and roots. These plant parts possesses some pleasant smell and it has been observed that this pleasant smell is actually due to the presence of convinced highly volatile oils known as essential oils. Essential oils are complex mixtures of volatile natural compounds, biosynthesized by many aromatic plants.

Essential oils have been used for thousands of years. The ancient civilizations of Mesopotamia, more than 5,000 years ago, had machines for obtaining essential oils from plants. Essential oils were the primary source of perfumes for the ancient civilizations of Egypt, India, Greece, and Rome. Essential oils have been found in 3,000-year-old tombs in the Pyramids, and early Greek physicians, including Hippocrates, mentioned aromatic plant essences and oil massages for their healing and mood-enhancing qualities. The Romans associated essential oils and their fine aromas with wealth and success. Ayurvedic medicine, the world's oldest healing system, has long recommended essential oil massage as a health treatment for many conditions.

Essential oils (EOs) are very interesting natural plant products and among other qualities they possess various biological properties. The term "biological" comprises all activities that these mixtures of volatile compounds exert on humans, animals, and other plants. Due to their diverse biological properties, plant volatiles are widely used as food additives to improve flavour and/or taste, and as preservatives to prevent growth of food-borne bacteria and fungi, thereby extending the shelf life of processed foods [1, 2]. Many essential oils contain isoprenoids. The essential oils are volatile liquids, mostly insoluble in water, but freely soluble in alcohol, ether, and vegetable and mineral oils. They are usually not oily to the touch [3].

OCCURRENCE:

Essential oils generally occurred from the different parts of the plant such as flowers, fruits, leaves, roots, seeds, and bark. In flowers (e.g. Rose, Jasmine, lavender oils), leaves (e.g. mint, Ocimum species, and lemongrass oils), bark (e.g. cinnamon oil), wood (e.g. cedar, sandal, and pine), roots (e.g. angelica), and seeds (e.g. fennel, coriander, caraway, dill, nutmeg), fruits (orange, lemon oils),

rhizomes (e.g. ginger, curcuma) and gums or oleoresin exudations (e.g. balsam of Peru).

A plant produces its essential oil in the protoplasm of its cells and is stored as micro droplets in glands. After diffusing slowly through the wall of the gland, these droplets spread out on the surface of the plant before evaporating and filling the air with perfume.

The oil is an excreted product, rich in energy and chemically very active; therefore, it is somewhat surprising for the plant to expel such an amount of energy without making use of it. The function of the essential oil in a plant is not well understood. An odour of flower helps in natural selection by acting as attractants for certain insects. Leaf oils, wood oils, and root oils may provide protection against plant parasites or depredations by animals. Oleo resinous exudations that appear when the trunk of a tree is injured prevent loss of sap and act as a protective seal against parasites and disease organisms. Few essential oils are involved in plant metabolism, and some investigators maintain that many of these materials are simply waste products of plant biosynthesis [4].

HISTORY OF ESSENTIAL OIL:

The history of essential oils related to the countries like ancient India, Persia, Egypt and the Europeans countries Greece and Rome which extensively dealt in odoriferous oils and ointments with their neighbouring countries. Most probably these products were extracts prepared by placing flowers, roots and leaves in fatty oils.

In most ancient cultures, aromatic plants or their resinous products were used directly. Only in the golden age of Arab culture, distillation techniques were developed for the essential oils. The Arabs were the first to distil ethyl alcohol from fermented sugar, thus providing a new solvent for the extraction of essential oils in place of the fatty oils that had probably been used for several millennia [4].

The knowledge of distillation spread to Europe during the Middle Ages, and during 12^{th} century isolation of essential oils by distillation was carried out. The distilled products, oils of cedar wood, rose, rosemary, incense, turpentine, cinnamon, benzoin [5] became a specialty of the European medieval pharmacies in 15^{th} century.

The development of the modern perfume industry started in France, the world centre of perfumery [4]. Perfumes are fragrant substances, generally of complex

composition, which gratify the sense of smell. Perfumery is the art of production of perfumes by compounding fragrant substances and in association with cosmetics [6].

FIRST SYSTEMATIC INVESTIGATION:

The first systematic investigations of constituents from essential oils may be attributed to the French chemist M. J. Dumas (1800–1884) who analyzed some hydrocarbons and oxygen as well as sulphur and nitrogen-containing constituents. He published his results in 1833. The French researcher M. Berthelot (1859) characterized several natural substances and their rearrangement products by optical rotation [7].

However, the most important investigations have been performed by O. Wallach, an assistant of Kekule. At that time, hydrocarbons occurring in essential oils with the molecular formula $C_{10}H_{16}$ were known, which had been named by Kekule terpenes because of their occurrence in turpentine oil. Constituents with the molecular formulas $C_{10}H_{16}O$ and $C_{10}H_{18}O$ were also known at that time under the generic name camphor and were obviously related to terpenes.

O. Wallach realized that several terpenes described under different names according to their botanical sources were often, in fact, chemically identical. He, therefore, tried to isolate the individual oil constituents and to study their basic properties; He employed his highly qualified co-workers Hesse, Gildemeister and others to separate essential oils and performed reactions with inorganic reagents like hydrochloric acid, oxides of nitrogen, bromine, and nitrosyl chloride to characterize the obtained individual fractions. In 1891, Wallach characterized the terpenes pinene, camphene, limonene, dipentene, phellandrene, terpinolene, fenchene, and sylvestrene, which has later been recognized to be an artifact [7].

During 1884–1914, Wallach wrote about 180 articles that are summarized in his book Terpene and Campher (Wallach, 1914) compiling all the knowledge on terpenes at that time and already in 1887 he suggested that the terpenes must be constructed from isoprene units. In 1910, he was honoured with the Nobel Prize for Chemistry "in recognition of his outstanding research in organic chemistry and especially in the field of alicyclic compounds [8].

In addition to Wallach, the German chemist A.von Baeyer in 1905 for his contributions to several dyes, the investigations of polyacetylenes and theoretical chemistry; he was awarded the Nobel Prize for Chemistry "in recognition of his

contributions to the development of Organic Chemistry and Industrial Chemistry, by his work on organic dyes and hydroaromatic compounds."

The frequently occurring acyclic monoterpenes geraniol, linalool, citral, and soon have been investigated by F. W. Semmler and the Russian chemist G. Wagner (1899) who recognized the importance of rearrangements for the elucidation of chemical constitution, especially the carbon-to-carbon migration of alkyl, aryl, or hydride ions, a type of reaction that was later generalized by H. Meerwein (1914) as Wagner–Meerwein rearrangement [9].

L. Ruzicka in Zurich, Switzerland; in 1939, was honoured in recognition of his outstanding investigations with the Nobel Prize in chemistry for his work on "polymethylenes and higher terpenes [10].

COMMERCIAL IMPORTANCE:

Essential oils have become a central part of everyday life. Essential oils are used in an enormous ways for many different reasons. They have a reflective effect on the central nervous system, relieving depression and anxiety, reducing stress, and relaxing. Many essential oils are used in perfumery. It takes kilogram of flowers to make small amount of essential oil. Moreover essential oil is utilized as aromatherapy which is a form medicine. Many essential oil often diluted and sometimes the oil is adulterated with synthetic chemicals.

Commercially, essential oils are used in three primary ways:

- **Flavours:** Food flavourings, as feed additives, as flavouring agents by the cigarette industry
- **Pharmaceuticals:** Balneology, massage, and homeopathy
- **Odorants:** Use in cosmetics, perfumes, air fresheners and deodorizers, soaps, detergents, and miscellaneous industrial products ranging from animals feed to insecticides to paints.

A more specialized area will be in the fields of aromatherapy. In recent years, the importance of essential oils as biocides and insect repellents has led to a more detailed study of their antimicrobial potential. Essential oils are also good natural sources of substances with commercial potential as starting materials for chemical synthesis.

BIOLOGICAL APPLICATION:

Essential oil finds an ample important application in pharmacological activity. Activities which are not directly related to central or autonomic nervous system and for which the molecular mechanisms are not significantly important, for example, anti oxidative effects, anticancer properties, penetration enhancing activities etc.

Anticancer Properties: A very promising field of treatment with EOs is their application against tumours. The main constituent of the EO of sweet orange peel oil (*Citrus sinensis*, Rutaceae) *d*-limonene as well as Perillyl alcohol has been found to have active against the tumours cell at very lower doses [11] .

Antiphlogistic Activity: Processes by which the body reacts to injuries or infections are called inflammations. EOs acts as a mediator against inflammations. The volatile oil obtained by steam distillation of the wood of *Cedrus* species shows a great anti-inflammatory and analgesic effect inhibition of carrageenin- induced rat paw edema [12].

Anti oxidative Properties: Free radicals are antagonistic, unstable, and highly reactive atoms, molecules or compounds because they carry a single electron and they attack other molecules to become stable, thereby changing their properties and making disorders inside possible. The EOs obtained from *Mentha piperita* L. and *Myrtus communis* L serve as an antioxidant agent in the lipid peroxidation inhibitory activities assessed by the b-carotene bleaching tests [13].

Pain Reliever: A numbers of conditions have been treated with essential oils with varying biological activities, such as antispasmodic, anti-inflammatory, anti arthritic and so on. They all share a common effect, that of pain relief.

Headache The use of peppermint and eucalyptus essential oils helps to relive the headache by affecting the neurophysiological, psychological parameters [14].

Osteoarthritis: A blend of *Zingiber officinale* (1%) and *Citrus sinensis* (0.5%) essential oils were used to severe knee pain caused by osteoarthritis [15].

EXTRACTION OF ESSENTIAL OIL:

Essential oils are the important plant products, generally of complex composition comprising the volatile principles contained in the plant and the more or less modified during the preparation process [16]. The oil droplets being stored in the oil glands can be removed by either accelerate diffusion through the cell wall or crush the cell wall. The adopted techniques depend on the part of the plants where the oil is to be extracted, the stability of the oil to heat and susceptibility of the oil constituents to chemical reactions [17].

Common techniques used for the extraction of essential oils are;

- Hydro distillation
- Hydro diffusion
- Effleurage
- Cold pressing
- Steam distillation
- Solvent extraction
- Microwave Assisted Process (MAP)
- Carbon dioxide extraction

ANALYTICAL TECHNIQUES FOR ESSENTIAL OIL:

The analytical methods play a pivotal role in analysing the essential oil compositions, identification and their structure elucidation.

The commonly applying techniques for analysing essential oil components include chromatographic methods such as GC (Gas Chromatography), HPLC (High Performance Liquid Chromatography), and HPTLC (High Performance Thin Layer Chromatography) for their individual separation.

The hyphenated techniques means instrumental on-line coupling of chromatographic separation devices like coupling of GC with Mass spectrometry (MS), GC-quadruple mass spectrometry with electron ionization (EI) or with Chemical ionization (CI), GC-ion trap tandem MS, Gas chromatography with Fourier transform infrared spectrometry (GC-FT-IR), GC-NMR as well as coupling of HPLC with MS. The main advantages of the hyphenated techniques are it provides more information related to the structure of the separated components and its identification. The hyphenated methods are the powerful and pragmatic tools for identifying components of complex mixtures.

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SECTION II

REVIEW

OF

LITERATURE

1.2.1.1 ESSENTIAL OIL STUDY:

Essential oils are volatile, natural, complex compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolites. They are usually obtained by steam or hydro-distillation. They are known for their antiseptic, i.e. bactericidal, virucidal and fungicidal, and medicinal properties and their fragrance; they are used in embalmment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthesic remedies. Up to the present day, these characteristics have not changed much except that more is now known about some of their mechanisms of action, particularly at the antimicrobial level.

In nature, essential oils play an important role in the protection of the plants as antibacterial, antiviral, antifungal, insecticides and also against herbivores by reducing their appetite for such plants. They also may attract some insects to favour the dispersion of pollens and seeds, or repel undesirable others.

Essential oils are extracted from various aromatic plants generally localized in temperate to warm countries like Mediterranean and tropical countries where they represent an important part of the traditional pharmacopoeia. They are liquid, volatile, limpid and rarely coloured, lipid soluble and soluble in organic solvents with a generally lower density than that of water. They can be synthesized by all plant organs, i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes.

There are several methods for extracting essential oils. These may include use of liquid carbon dioxide or microwaves, and mainly low or high pressure distillation employing boiling water or hot steam.

Due to their bactericidal and fungicidal properties, pharmaceutical and food uses are more and more widespread as alternatives to synthetic chemical products to protect the ecological equilibrium. In those cases, extraction by steam distillation or by expression, for example for Citrus, is preferred. For perfume uses, extraction with lipophilic solvents and sometimes with supercritical carbon dioxide is favoured. Thus, the chemical profile of the essential oil products differs not only in the number of molecules but also in the stereochemical types of molecules extracted, according to the type of extraction, and the type of extraction is chosen according to the purpose of the use. The extraction product can vary in quality, quantity and in composition according to climate, soil composition, plant organ, age and vegetative

cycle stage (Masotti et al., 2003; Angioni et al., 2006). So, in order to obtain essential oils of constant composition, they have to be extracted under the same conditions from the same organ of the plant which has been growing on the same soil, under the same climate and has been picked in the same season. Most of the commercialized essential oils are chemo typed by gas chromatography and mass spectrometry analysis. Analytical monographs have been published (European pharmacopoeia, ISO, WHO, Council of Europe; Smith et al., 2005) to ensure good quality of essential oils.

Essential oils have been largely employed for their properties already observed in nature, i.e. for their antibacterial, antifungal and insecticidal activities. At present, approximately 3000 essential oils are known, 300 of which are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries.

Essential oils or some of their components are used in perfumes and make-up products, in sanitary products, in dentistry, in agriculture, as food preservers and additives, and as natural remedies. For example, d-limonene, geranyl acetate or d-carvone are employed in perfumes, creams, soaps, as flavour additives for food, as fragrances for household cleaning products and as industrial solvents.

Moreover, essential oils are used in massages as mixtures with vegetal oil or in baths but most frequently in aromatherapy. Some essential oils appear to exhibit particular medicinal properties that have been claimed to cure one or another organ dysfunction or systemic disorder (Silva et al., 2003; Hajhashemi et al., 2003; Perry et al., 2003).

Owing to the new attraction for natural products like essential oils, despite their wide use and being familiar to us as fragrances, it is important to develop a better understanding of their mode of biological action for new applications in human health, agriculture and the environment. Some of them constitute effective alternatives or complements to synthetic compounds of the chemical industry, without showing the same secondary effects (Carson and Riley, 2003).

1.2.1.2 CHEMICAL COMPOSITION:

Essential oils are very complex natural mixtures which can contain about 20–60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20–70%) compared to others components present in trace amounts. For example, carvacrol (30%) and thymol (27%) are the major components of the *Origanum compactum* essential oil, linalol (68%) of the *Coriandrum sativum* essential oil, a- and b-thuyone (57%) and camphor (24%) of the *Artemisia herba-alba* essential oil, 1,8-cineole (50%) of the *Cinnamomum camphora* essential oil, a-phellandrene (36%) and limonene (31%) of leaf and carvone (58%) and limonene (37%) of seed *Anethum graveolens* essential oil, menthol (59%) and menthone (19%) of *Mentha piperita* (=Mentha· piperita) essential oil. Generally, these major components determine the biological properties of the essential oils. The components include two groups of distinct biosynthetical origin (Croteau et al., 2000; Betts, 2001; Bowles, 2003; Pichersky et al., 2006). The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, all characterized by low molecular weight.

1.2.1.3 BIOLOGICAL EFFECT:

Cytotoxicity:

Because of the great number of constituents, essential oils seem to have no specific cellular targets (Carson et al., 2002). As typical lipophiles, they pass through the cell wall and cytoplasmic membrane, disrupt the structure of their different layers of polysaccharides, fatty acids and phospholipids and permeabilize them. Cytotoxicity appears to include such membrane damage. In bacteria, the permeabilization of the membranes is associated with loss of ions and reduction of membrane potential, collapse of the proton pump and depletion of the ATP pool (Knobloch et al., 1989; Sikkema et al., 1994; Helander et al., 1998; Turina et al., 2006). Essential oils can coagulate the cytoplasm (Gustafson et al., 1998) and damage lipids and proteins (Ultee et al., 2002; Burt, 2004). Damage to the cell wall and membrane can lead to the leakage of macromolecules and to lysis (Lambert et al., 2001; Oussalah et al., 2006).

This cytotoxic property is of great importance in the applications of essential oils not only against certain human or animal pathogens or parasites but also for the preservation of agricultural or marine products.

Examples of essential oils tested for their cytotoxic capacities on standard organisms include *Pinus densiflora, Pinus koraiensis, and Chamaecyparis obtuse* on organism *Salmonella typhimurium, Listeria monocytogenesis, Escherichia coli, Staphylococcus aureus , Klebsiella pneumonia and Candida albicans* in the concentration range of 50µL of dilutions 1/2, 1/4, 1/8, 1/16 on filter paper discs (Hong et al. 2004).

Phototoxicity:

Some essential oils contain photoactive molecules like furocoumarins. For instance, *Citrus bergamia* essential oil contains psoralens which bind to DNA under ultraviolet A light exposure producing mono- and bi-adducts that are cytotoxic and highly mutagenic (Averbeck et al., 1990).

1.2.1.4 MEDICINAL AND FUTURE MEDICINAL APPLICATION:

The cytotoxic capacity of the essential oils based on a pro-oxidant activity can make them excellent antiseptic and antimicrobial agents for personal use, i.e. for purifying air, personal hygiene, or even internal use via oral consumption, and for insecticidal use for the preservation of crops or food stocks A big advantage of essential oils is the fact that they are usually devoid of long-term genotoxic risks. Many radical producing agents are in fact used in antitumor treatments. In the case of essential oils, radical production could be very well controlled and targeted without presenting by itself any toxic or mutagenic side-effects to healthy tissues. Essential oils or their active constituents could be included in vectorized liposomes (Sinico et al., 2005; Lai et al., 2006; Fang et al., 2006) that would allow bettering defining the quantities applied. Thus, essential oils could make their way from the traditional into the modern medical domain.

1.2.1.5 ANTI-ARTHRITIC STUDY:

Arthritis is an auto immune disorder characterized by pain, swelling and stiffness. Its prevalence depends upon age. It is an inflammation of synovial joint due to immune-mediated response. Rheumatoid arthritis has 19th century roots and a 20th century pedigree.

Rheumatoid arthritis is characterised by persistent synovitis, systemic inflammation and auto antibodies. Rheumatoid arthritis (RA) is a chronic, inflammatory, systemic autoimmune disease that affects about 1% of the general population in Western countries and is two to three times more common in women than in men. It is characterized by both local and systemic inflammation with elevated plasma concentration of pro-inflammatory cytokines, such as interleukins - 6 (IL-6), interleukin 1b (IL-1b), tumour necrosis factor-alfa (TNF-a), and acute phase proteins.

Conventional treatments for RA, including Non-steroidal Anti-inflammatory Drugs (NSAID's), disease modifying anti-rheumatoid drugs (DMARD's) and corticosteroids, aim to reduce the patient's pain and joint inflammation, minimize loss of function and decrease the progression of joint damage. Much of the joint damage that ultimately results in disability begins early in the course of the disease. In one study, for example, more than 80 percent of patients with rheumatoid arthritis of less than two years duration had joint space narrowing on plain radiographs of the hands and wrists, while two-thirds had erosions.

An appreciation of the pathogenic mechanisms of rheumatoid arthritis and the poor outcomes with conventional therapy led to the concept of effective treatment of newly diagnosed or early aggressive disease to suppress ongoing inflammation and prevent joint injury.

All anti inflammatory drugs are not anti-arthritic because it does not suppress T-cell and B-cell mediated response. Rheumatoid arthritis is associated with poor nutritional status in relation to various nutrients due to not only because of increased requirements and reduction in their absorption but also due to NSAID's, DMARD's and corticosteroids prescribed to alleviate symptoms of this disease.

List of anti-arthritic medicinal plants:

Botanical Name	**Family**	**Parts Used**	**References**
Aristrolochia bracteata	Aristrolochiaceae	Whole plant	Havagiray R.chitme and nitin et al
Boswelia serrata	Burseraceae	Gum resin	Mishra
Centenella asiatica	apiaceae	Fresh whole plant	Seema vangalapati et al
Glycerriza glabra	Leguminosae	Rhizomes	Mishra
Merremia tridentate	Convolvulaceae	Root and aerial part	Gopala krishnan et al
Vitex negundo	Verbenaceae	Leaves	Ramesh petchi. R. et al
Mangifera indica Linn.	Anacardiaceae	Leaves, bark	Garrido
Hemidusmus indicus Linn.	Asclepiadaceae	Roots	Shaikh
Zingiber officinale	Zingiberaceae	Roots	Rehman R et al
Anisomeles malabarica	Lamiaceae	leaves	Lavanya et al
Withania somnifera Linn.	Solanaceae	Root	Mirjalili MH et al
Piper nigrum Linn.	Piperaceae	Bud	Bang JS et al
Aloe barbadensis	Liliaceae	Whole plant	Joshph B et al
Calotropis Procera Linn.	Asclepiadaceae	Latex	Kumar VL

1.2.1.6 SELECTION OF PLANTS:

***Anisomeles malabarica* R.Br**

Family: Lamiaceae

Anisomeles malabarica R.Br is a well known medicinal plant in Ayurvedic and Siddha medicine. *Anisomeles malabarica* are used against convulsions, for dyspepsia in intermittent fevers, colic, boils, tetanus, inflammation, cough, cold, stomach-ache, itches and in uterine affections.

Plant description:

Anisomeles malabarica is an aromatic, densely pubescent, perennial herb, 1.2–2.0 m in height belonging to the family Lamiaceae. It is commonly found in Western Ghats from Maharashtra to Karnataka, Andhra Pradesh, Kerala and Tamil Nadu (Hardin et al, 2009). It is commonly called by various names throughout the country as mentioned below.

Common name:

English:	Malabar catmint,
Hindi:	Chodara
Malayalam:	Peyameratti
Sanskrit:	Vaikuntha

The leaves of the plant are simple, thick, velvety, petiolate, lanceolate to oblong, 6.3 - 10.0 cm × 2.0–4.5 cm, acute apex, rounded at the base with bluntly serrate margin. The lamina is tomentose and clothed with pubescent hairs on both surfaces. The veins are prominent on lower side; upper surface is green while the lower surface is pale green (Mariapackiam S. Et al, 2007). Flowers are pale purple in color, occasionally white, in axillary whorls of dense cymes; nut lets ellipsoid, smooth, compressed, 3–4 mm long (Raja Boobalan et al, 2010). The plant has a tap root system. Roots have a dirty yellowish cork surface and whitish wood. The roots are odourless and tasteless. Fracture of the wood is fibrous (Kapoor V.P. et al, 1975). The stem is obtusely quadrangular, ash grey in color. It is densely tomentose or thickly woody and slightly branched. The inner surface is light brown in older stem. It also shows brown streaks on its outer surface (Choudhary N et al.2011).

Phytochemical profile:

Phytochemical examination revealed the presence of essential oils with citral as the chief chemical constituent. The petroleum ether extract of plant contains a triterpenic acid known as betulinic acid (Gupta A.K. et al 2004) while the hexane extract consists of β-sitosterol and diterpenoids (ovatodiolide and anisomelic acid) (Purushothaman K.K. et al.1975)**.** The aerial parts contain anisomelolide, malabaric acid, 2-acetoxymalabaric acid, anisomelyl acetate, anisomelol and anisomelin (Devi G et al.1979).The seeds are mainly comprised of proteins and amino acids, viz., aspartic acid, threonine, serine, glutamine, proline, glycine, alanine, valine, metheonine, isoleucine, leucine, tryptophan, phenylalanine, histidine, lysine and arginine (Zahir A.A.2010).

Pharmacological activity:

Antifertility and Antispasmodic

The ethanolic extract of the plant (excluding root) showed spermicidal effect on respiration and antispasmodic in experimental animals (Setty B.S. et al. 1977)

Anticancer and Diuretic

It also possesses anticancer activity against P388 lymphocytic leukemia in mice and diuretic activity in rat (Sharma R.N. et al. 1979) The ethanolic extract at an oral dose of 100 mg kg−1 exhibited a significant protective effect by reduce liver and serum levels of total protein, GPT, GOT, ACP and ALP as compared to DEN induced mice (Jeyachandran R. et al. 2007).

Antimicrobial

The ethanolic extract of leaves showed antibacterial activity against strains of *Echerichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus*. Lymphocyte leukaemia in mice and diuretic activity in rat (Vijaya K.R.1998).

Anticonvulsant Activity

Chloroform and ethyl acetate extracts of *A. malabarica* leaves are effective in pentylenetetrazole- and maximal electroshock-induced convulsions in rats (Sitholey R.V. 1971). We have also demonstrated that the ethyl acetate extract (flavonoid fraction) of *A. malabarica* is effective in experimental models of convulsions (Dev S. 1997).

Antifeedant Activity

Hexane, ethyl acetate, acetone, and methanol extracts of *A. malabarica* (L.) Sims leaves was found to have antifeedant activity (Evans W.C. et al. 2006).

Hypocholesterolemic Activity

The ethanolic extract of the aerial parts of this plant has been studied against the standard drag lipocor tablets and rabbits have been used as experimental animals (Kamaraj C. 2010).

Anthelmintic Activity

Ethyl acetate, acetone and methanol extract of leaf, bark, and seed of *A. malabarica* (L.) R. Br were tested against the parasitic nematode of small ruminants H. contortus using egg hatch assay (EHA) and larval development assay (LDA) (Kannabiran B et al 1972).

Antioxidant Activity

The methanolic extract of leaves has shown in vitro antioxidant activity (Rajwar G.S. 1983).

Insecticidal Activity

Ethanolic extract of *A. malabarica* was tested against the larvae of Spodoptera litura (Mehrorta S et al, 2005).

Adulticidal Activity

Hexane, chloroform, ethyl acetate, and acetone leaves extracts of *A. malabarica* have shown the adult emergence inhibition and adulticidal activity (Zahir A.A.2010)

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SECTION III

MATERIAL COLLECTION AND IDENTIFICATION

1.3.1 *ANISOMELES MALABARICA* R.BR.

1.3.1.1 INTRODUCTION:

Anisomeles malabarica R.BR is an aromatic, densely pubescent, perennial herb, 1.2–2.0 m in height belonging to the family Lamiaceae. The leaves of the plant are simple, thick, velvety, petiolate, lanceolate to oblong, 6.3–10.0 cm × 2.0–4.5 cm, acute apex, rounded at the base with bluntly serrate margin (Figure 1). Flowers are pale purple in colour, occasionally white, smooth, compressed, 3–4 mm long (Figure 2).

1.3.1.2 GEOGRAPHICAL DISTRIBUTION:

It is commonly found in Western Ghats from Maharashtra to Karnataka, Andhra Pradesh, Kerala and Tamil Nadu. [1]

1.3.1.3 TAXONOMICAL CLASSIFICATION:

Kingdom	**Plantae**
Subkingdom	*Viridaeplantae*
Phylum	*Tracheophyta*
Class	*Magnoliopsida*
Subclass	*Lamiidae*
Order	*Lamiales*
Family	*Lamiaceae*
Genus	*Anisomeles*
Species	*Malabarica R.Br.*
Botanical name	***Anisomeles malabarica*** R.Br.

1.3.1.4 VERNACULAR NAMES:

English: Malabar Catmint

Hindi: Chodara

Sanskrit: Vaikuntha

Ayurvedic: Sprikkaa. [2]

Siddha/Tamil: Irattaipeyameratti. [3]

Malayalam: Karimtumba.

1.3.1.5 PART OF THE PLANT USED:

Leaves and flowers of *Anisomeles malabarica* R.BR were used for the present research work (Figure 1and 2).

1.3.1.6 PHYTOCHEMISTRY:

Phytochemical examination revealed the presence of essential oils with citral as the chief chemical constituent. The volatile oil content is the highest in flowers (0.07%) followed by leaves (0.025%). The volatile oil content is the least in root and stem (0.005%). The petroleum ether extract of plant contains a triterpenic acid known as betulinic acid [4] while the hexane extract consists of β-sitosterol and diterpenoids (ovatodiolide and anisomelic acid) [5]. The aerial parts contain anisomelolide, malabaric acid, 2-acetoxymalabaric acid, anisomelyl acetate, anisomelol and anisomelin. [1, 6, 7]

1.3.1.7 COLLECTION:

The whole plant of *Anisomeles malabarica* R.Br. was collected twice (June and November) in a year, from Dindigul district region of Tamil Nadu, India.

1.3.1.8 AUTHENTICATION:

The plant was botanically authenticated. A voucher specimen (MVS-1) of the plant has been deposited at the herbarium of the Botanical Survey of India, Pune. A copy of the authenticated herbarium (Figure 3) and the authentication certificate is given below (Figure 4)

Figure 1: ANISOMELES MALABARICA R.BR.

Figure 2: ANISOMELES MALABARICA FLOWERS

Figure 3: HERBARIUM PICTURE

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भारतीय वनस्पति सर्वेक्षण
पश्चिमी क्षेत्रीय केंद्र
७, कोरेगांव रोड, पुणे- ४११ ००१

No. BSI/WRC/Tech./2014/ Date 28-02-2014

CERTIFICATE

This is to certify that the plant specimen brought by **Mr. Mohmad Vasim Sheikh**, student of Ph.D. from **Ramnarain Ruia College, Mumbai** is identified as:

Number	Name	Family
MVS – 1	*Anisomeles malabarica* R.Br.	Lamiaceae

28/02/14
(J. Jayanthi)
For Scientist 'C' & H.o.O

वैज्ञानिक 'सी'
Scientist C
भारतीय वनस्पति सर्वेक्षण
Botanical Survey of India
पश्चिमी क्षेत्रीय केन्द्र, पुणे-1
Western Regional Centre, Pune

Figure 4: AUTHENTICATION CERTIFICATE

1.3.1.9 REFERNCES:

1. Neeraj Choudhary, Amit Kumar, Naveen Bimal, B.V Krishna Reddy. "A Review of Phytochemistry and Biological Activities of Anisomeles malabarica". Research & Reviews: Journal of Herbal Science Volume 1, Issue 1, Pages 1-9, 2012
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CHAPTER II
QUALITY CONTROL

INTRODUCTION TO QUALITY CONTROL:

Quality control (QC) is the process of ensuring that the operational techniques and activities used in an analytical laboratory provide results suitable for the intended purpose. Quality control programs are key components which broadly include all analytical protocols in all areas of analysis, including environmental, pharmaceutical, and forensic testing, and used to bring a system into statistical control.

The most important feature of quality control is a set of written directives describing all relevant laboratory-specific, technique-specific, sample-specific, method-specific, and protocol-specific operations. [1, 2, 3]

QUALITY CONTROL FOR HERBAL MEDICINAL PLANTS:

The use of herbs as medicine is the oldest form of healthcare known to humanity and has been used in all cultures throughout history [4] and has become a topic of global importance. Current scenario suggests that, in many developing countries such as India, China, a major part of the population follows the traditional practices and medicinal plants to meet primary health care needs. Although modern medicine have been available in these countries, herbal medicines (phytomedicines) have often maintained popularity for historical and cultural reasons as a result many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs.

Furthermore, in most countries the herbal medicines market is poorly regulated, and herbal products are often neither registered nor controlled. Assurance of the safety, quality, and efficacy of medicinal plants and herbal products has now become a key issue in industrialized and in developing countries. Both the general consumer and healthcare professional's need up to date, authoritative information on the safety and efficacy of medicinal plants. [5]

In order to have a good coordination between the quality of raw materials, in process materials and the final products, it has become essential to develop reliable, specific and sensitive quality control methods using a combination of classical and modern instrumental method of analysis.

Standardization is an essential measurement for ensuring the quality control of the herbal drugs. [6]

"Standardization" expression is used to describe all measures, which are taken during the manufacturing process and quality control leading to a reproducible quality.

The quality control of phytopharmaceuticals may be defined as the status of a drug, which is determined either by identity, purity, content or assay, and other chemical, physical or biological properties, or by the manufacturing process.

Quality control is based on three important pharmacopeial definitions:

- Identity: This quality parameter shows the precise identification of the herbs selected for the medicinal purposes.
- Purity: Presence or absence of contaminants or impurity in the form of other herbs imply there purity.
- Content or assay: Analysing the defined limits for the active constituents.

As a result, ICDRA and WHO committees are now publishing monographs for the plants possessing the therapeutic and medicinal important. These guidelines and monograph gives valuable information related to medicinal plants.

WHO GUIDELINES FOR QUALITY STANDARDIZED HERBAL DRUGS:

Guidelines for the assessment of herbal medicines were subsequently prepared by World Health Organisation (WHO) and adopted by the sixth International Conference of Drug Regulatory Authorities (ICDRA) in Ottawa, Canada, in 1991. [7] WHO has set certain standards for herbal drugs. It involves the following parameters.

BOTANICAL PARAMETERS

- Sensory evaluation: Includes visual microscopy/touch/odour/taste
- Foreign matter: Includes foreign plants, foreign animals, foreign minerals, etc.
- Microscopy: Includes histological observation and measurements

PHYSICO-CHEMICAL PARAMETERS

- Ash values: Total, acid-insoluble, water-soluble
- Extractive values: In hot water, cold water and ethanol
- Moisture content and volatile matter: Loss on drying (LOD), azeotropic distillation
- TLC/HPTLC finger print

PHARMACOLOGICAL PARAMETERS

- Bitterness value: Unit equivalent bitterness of standard solution of quinine hydrochloride
- Haemolytic property: On ox blood by comparison with standard reference solution of saponin
- Astringent property: Tannins that bind to standard Frieberg Hide powder
- Swelling index: In water
- Foaming index: Foam height produced by 1gm material under specific conditions

TOXICOLOGICAL PARAMETERS

- Arsenic: Stain produced on $HgBr_2$ paper in comparison to standard stain
- Pesticide residues: Includes total organics chloride and total organic phosphorus
- Heavy metals: Like cadmium and lead
- Microbial contamination: Total viable aerobic count of pathogens: *Enterobacteriaceae, E.coli, Salmonella, P.aeruginosa, S.aureous*
- Aflatoxins: By TLC using standard aflatoxins (B1, B2, G1 and G2)
- Radioactive contamination

This chapter include three sections. The Section I deal with the proximate analysis details about how the quality control methods used for herbal medicinal plant materials. Section II involves the optimization of extraction conditions and finally Section III deals with qualitative analysis of the different solvent extract for the major phytoconstituents present in the plants.

SECTION I

PROXIMATE ANALYSIS

2.1 PROXIMATE ANALYSIS:

It gives valuable information and help to access the quality of the sample. It provides information such as moisture content, ash content, volatile matter content etc.

Plants contain almost 90% of the water and remaining organic matter like as human body which is build up of water and organic content. Checking moisture content helps to reduces error in the examination of the actual weight of the drug materials and provides the stability of the drug against the degradation.

Ash is the inorganic residue remaining after water and organic matter have been removed by heating. Ash value provides a measure of total amount of minerals present in the herbal drugs, plant materials and in food. It gives the important information related to minerals which may cause some pharmacological effect on living being.

Extractive values give the indicative weights of the extractable chemical constituent present in the crude drug under different solvents environments. The use of single solvent can be the means of providing preliminary information on the quality of a particular drug sample.

The various physical chemical parameters were applied for the selected medicinal plants.

2.1.1 PHYSICO-CHEMICAL ANALYSIS:

2.1.1.1 DETERMINATION OF ASH VALUES

A) TOTAL ASH

PROCEDURE:

1. Approximately one grams of dried powder and paste of each plant were taken in the previously weighed silica crucible separately.
2. The incineration was carried out at temperature not exceeding 450ºC in muffle furnace till free from carbon, indicated by colour change.
3. The white ash obtained was cooled in desiccators and weighed and the procedure repeated till constant weight was obtained.
4. The percentage of total ash was calculated and tabulated on the basis of sample taken initially.

B) ACID INSOLUBLE ASH

PROCEDURE:

1. The crucible containing total ash was transferred in a beaker and 25.0mL of hydrochloric acid (2M) was added.

2. The beaker content was covered with a watch-glass and boiled gently for 5 minutes. The watch-glass was rinsed with 5.0mL of hot water and this liquid was added to the crucible.

3. The insoluble matter was collected on an ash less Whatman filter paper No. 41 and it was washed with hot water until the filtrate become neutral.

4. The filter paper containing the insoluble matter was transferred to the original crucible; it was dried on a hot plate and ignited in a furnace to constant weight. The residue was allowed to cool and then weighed.

5. The percentage of acid insoluble ash was calculated and tabulated on the basis of sample taken initially.

C) WATER SOLUBLE ASH

PROCEDURE:

1. To the crucible containing the total ash, 25.0mL of water was added and boiled for 5 minutes.

2. The insoluble matter was filtered and collected on an ash less Whatman filter paper No. 41.

3. It was washed with hot water and dried on a hot plate ignited in a furnace to constant weight. The residue was allowed to cool and then weighed.

4. Weight of insoluble matter was subtracted from the weight of total ash. The percentage of water soluble ash was calculated and tabulated on the basis of sample taken initially.

2.1.1.2 MOISTURE CONTENT

LOSS ON DRYING

PROCEDURE:

1. Approximately two grams of dried powder and paste of each plant were taken in an evaporating dish and then dried in an oven at 110°C till constant weight was obtained.
2. The weight after drying was noted and loss on drying was calculated by using the following formula.
3. The percentage was calculated and tabulated on the basis of sample taken initially.

2.1.1.3 DETERMINATION OF EXTRACTIVE VALUES

PROCEDURE:

A) ETHANOL SOLUBLE EXTRACTIVE VALUE

1. Approximately two grams of dried powder and paste of each plant were macerated with 100.0mL of ethanol in a closed flask for 24 hours.

2. The flask was frequently shaken during first 6 hours and allowed to stand for 18 hours.

3. The content in the flask was filtered rapidly taking precautions against loss of ethanol.

4. Thereafter, it was filtered in a previously weighed empty beaker and the filtrate was evaporated to dryness at 80°C in thermostat and weighed.

5. The percentage of extractable matter was calculated and tabulated with reference to the sample taken initially.

B) WATER SOLUBLE EXTRACTIVE VALUE

The above same procedure was followed using water instead of ethanol. The percentage of extractable matter was calculated and tabulated with reference to the sample taken initially.

2.1.1.4 OBSERVATIONS:

TABLE 2.1.1.4.1

TOTAL ASH %: *ANISOMELES MALABARICA* R.BR LEAVES

SERIAL NO	WT. OF EMPTY CRUCIBLE (g)	WT. OF SAMPLE (g)	WT. OF CRUCIBLE + ASH (g)	TOTAL ASH (%)	MEAN %	STANDARD DEVIATION %
1	16.7587	1.0015	16.7843	2.56	2.45	0.077309
2	17.1724	1.0010	17.1971	2.47		
3	17.5803	1.0000	17.6042	2.39		
4	15.7926	1.0027	15.8164	2.37		
5	15.9223	1.0018	15.9475	2.52		
6	15.5907	1.0031	15.6148	2.40		

TABLE 2.1.1.4.2

TOTAL ASH %: *ANISOMELES MALABARICA* R.BR FLOWERS

SERIAL NO	WT. OF EMPTY CRUCIBLE (g)	WT. OF SAMPLE (g)	WT. OF CRUCIBLE + ASH (g)	TOTAL ASH (%)	MEAN %	STANDARD DEVIATION %
1	16.7588	1.0016	16.9018	14.23	14.70	0.360708
2	17.1723	1.0009	17.3223	14.99		
3	17.5805	1.0002	17.7275	14.69		
4	15.5909	1.0029	15.7399	14.86		
5	15.7924	1.0019	15.9354	14.30		
6	15.9221	1.0029	16.0731	15.10		

TABLE 2.1.1.4.3

ACID INSOLUBLE ASH %: *ANISOMELES MALABARICA* R.BR LEAVES

SERIAL NO	WT. OF EMPTY CRUCIBLE (g)	WT. OF SAMPLE (g)	WT. OF CRUCIBLE + ASH (g)	ACID INSOLUBLE ASH (%)	MEAN %	STANDARD DEVIATION %
1	15.7926	1.0027	16.7953	1.27	1.24	0.030551
2	15.9223	1.0018	15.9346	1.23		
3	15.5907	1.0031	15.6028	1.21		

TABLE 2.1.1.4.4

ACID INSOLUBLE ASH %: *ANISOMELES MALABARICA* R.BR FLOWERS

SERIAL NO	WT. OF EMPTY CRUCIBLE (g)	WT. OF SAMPLE (g)	WT. OF CRUCIBLE + ASH (g)	ACID INSOLUBLE ASH (%)	MEAN %	STANDARD DEVIATION %
1	16.7588	1.0016	16.8211	6.22	6.25	0.03
2	17.1723	1.0009	17.2352	6.28		
3	17.5805	1.0002	17.6430	6.25		

TABLE 2.1.1.4.5

WATER SOLUBLE ASH %: *ANISOMELES MALABARICA* R.BR LEAVES

SERIAL NO	WT. OF EMPTY CRUCIBLE (g)	WT. OF SAMPLE (g)	WT. OF CRUCIBLE + ASH (g)	WATER SOLUBLE ASH (%)	MEAN %	STANDARD DEVIATION %
1	16.7587	1.0015	16.7664	0.77	0.79	0.02
2	17.1724	1.0010	17.1805	0.81		
3	17.5803	1.0000	17.5882	0.79		

TABLE 2.1.1.4.6

WATER SOLUBLE ASH %: *ANISOMELES MALABARICA* R.BR FLOWERS

SERIAL NO	WT. OF EMPTY CRUCIBLE (g)	WT. OF SAMPLE (g)	WT. OF CRUCIBLE + ASH (g)	WATER SOLUBLE ASH (%)	MEAN %	STANDARD DEVIATION %
1	15.5909	1.0029	15.6122	2.12	2.12	0.055076
2	15.7924	1.0019	15.8130	2.06		
3	15.9221	1.0029	15.9438	2.17		

TABLE 2.1.1.4.7

LOSS ON DRYING %: *ANISOMELES MALABARICA* R.BR LEAVES

SERIAL NO	WT. OF EVAPORATING DISH (g)	WT. OF SAMPLE (g)	WT. OF DISH + RESIDUE (g)	LOD (%)	MEAN %	STANDARD DEVIATION %
1	68.659	2.0	68.788	6.45	6.23	0.202073
2	89.672	2.0	89.793	6.05		
3	75.568	2.0	75.692	6.20		

TABLE 2.1.1.4.8

LOSS ON DRYING %: *ANISOMELES MALABARICA* R.BR FLOWERS

SERIAL NO	WT. OF EMPTY EVAPORATING DISH (g)	WT. OF SAMPLE (g)	WT. OF DISH + RESIDUE (g)	LOD (%)	MEAN %	STANDARD DEVIATION %
1	68.663	2.0	68.713	2.50	2.77	0.236291
2	89.667	2.0	89.726	2.95		
3	75.568	2.0	75.625	2.85		

TABLE 2.1.1.4.9

ETHANOL SOLUBLE EXTRACTIVE VALUE %:

***ANISOMELES MALABARICA* R.BR LEAVES**

SERIAL NO	WT. OF EMPTY BEAKAR (g)	WT. OF SAMPLE (g)	WT. OF BEAKER + RESIDUE (g)	EXTRACTIVE VALUE (%)	MEAN %	STANDARD DEVIATION %
1	120.283	2.0	120.640	17.85	17.45	0.4
2	125.009	2.0	125.350	17.05		
3	113.212	2.0	113.561	17.45		

TABLE 2.1.1.4.10

ETHANOL SOLUBLE EXTRACTIVE VALUE %:

***ANISOMELES MALABARICA* R.BR FLOWERS**

SERIAL NO	WT. OF EMPTY BEAKAR (g)	WT. OF SAMPLE (gm	WT. OF BEAKER + RESIDUE (g)	EXTRACTIVE VALUE (%)	MEAN %	STANDARD DEVIATION %
1	120.661	2.0	120.7108	2.49	2.57	0.075056
2	139.566	2.0	139.6188	2.64		
3	115.213	2.0	115.2643	2.57		

TABLE 2.1.1.4.11

WATER SOLUBLE EXTRACTIVE VALUE %:

***ANISOMELES MALABARICA* R.BR LEAVES**

SERIAL NO	WT. OF EMPTY BEAKAR (g)	WT. OF SAMPLE (g)	WT. OF BEAKER + RESIDUE (g)	EXTRACTIVE VALUE (%)	MEAN %	STANDARD DEVIATION %
1	120.283	2.0	120.512	11.45	11.65	0.2
2	125.005	2.0	125.238	11.65		
3	113.215	2.0	113.452	11.85		

TABLE 2.1.1.4.12

WATER SOLUBLE EXTRACTIVE VALUE %:

***ANISOMELES MALABARICA* R.BR FLOWERS**

SERIAL NO	WT. OF EMPTY BEAKAR (g)	WT. OF SAMPLE (g)	WT. OF BEAKER + RESIDUE (g)	EXTRACTIVE VALUE (%)	MEAN %	STANDARD DEVIATION %
1	115.430	2.0	115.689	12.95	12.57	0.40104
2	110.661	2.0	110.904	12.15		
3	93.515	2.0	93.767	12.60		

2.1.1.5 RESULTS AND DISCUSSION:

The results obtained from the proximate analysis of physicochemical parameters for *Anisomeles malabarica* R.BR leaves & flowers were tabulated and the average values are expressed as percentage of air-dried material.

TABLE 2.1.1.5.1

PARAMETRES	***Anisomeles malabarica* R.BR**	
	Leaves (w/w)%	**Flowers (w/w)%**
Total ash	2.45	14.70
Acid insoluble ash	1.24	6.25
Water soluble ash	0.79	2.12
Loss on drying	6.23	2.76
Ethanol soluble extractive	17.45	2.56
Water soluble extractive	11.65	12.57

The physical constant evaluation of the powder is an important parameter in detecting adulteration or improper handling of drugs. The percentage of active chemical constituents in crude drugs is mentioned on air-dried basis. Therefore, the loss on drying of plant materials should be determined and the water content should also be controlled. The loss on drying of dry powder and paste of *Anisomeles malabarica* R.BR leaves & flowers showed the loss on drying 6.23 % and 2.76 % respectively were in limits suggested by Indian council of medical research (ICMR).

The total ash is particularly important in the evaluation of purity of drugs, i.e. the presence or absence of foreign inorganic matter such as metallic salts and/or silica. Low amount of total ash, acid insoluble ash and water soluble ash indicate that the inorganic matter and non-physiological matter such as silica is less in *Anisomeles malabarica* R.BR leaves & flowers.

The extractive values are useful to evaluate the chemical constituents present in the crude drug and also help in estimation of specific constituents soluble in a particular solvent. The variation in extractable matter in various solvents is suggestive of the fact that the formation of the bioactive principle of the medicinal plants is influenced by number of intrinsic and extrinsic factors. High alcohol soluble and water soluble extractive values reveal the presence of polar substance like phenols, tannins and glycosides.

2.1.1.6 REFERENCES:

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PHYTOCHEMICAL ANALYSIS

INTRODUCTION:

There are simple but standard chemical tests to detect the presence of alkaloids, tannins, saponins etc in a plant extract. Each time the operator is undertaking the plant phytochemical screening; there is always the need to carry out confirmatory tests because one can experience false-positive reactions in some non alkaloidal extracts. Plants are subject to physiological changes before extraction, if the plants are not extracted on the day of collection and this could invariably affect the phytochemical screening results.

2.1.2 PHYTOCHEMICAL ANALYSIS

2.1.2.1 PREPARATION OF EXTRACTS

The successive solvent extraction was performed by subjecting the dry powder and paste of the selected plants by various solvents in order of increasing polarity using sonication techniques.

2.1.2.2 QUALITATIVE ANALYSIS OF EXTRACTS

The extract obtained after successive solvent extractions were subjected to several chemical tests to check the presence or absence of particular plant constituents.

A) TEST FOR ALKALOIDS:

About 1.0mL of different solvent extract was taken in 5% HCl (2.0mL) solution and heated on water bath.

1. **Dragendorff's Test:** Plant Extract + Dragendroff reagent (Potassium iodide and Bismuth nitrate in acetic acid)
2. **Hager's Test:** Plant Extract + Saturated Picric acid solution
3. **Wagner's Test:** Plant Extract + Wagner reagent (Mercuric chloride and Potassium iodide in distilled water)
4. **Mayer's Test**: Plant Extract + Mayer reagent (Potassium and Iodine in distilled water)

Presence of orange red, yellow and reddish brown, creamy precipitates with respective reagents detected the presence of alkaloids.

B) TEST FOR FLAVANOIDS:

1. **Shinoda Test:** To 1.0mL extract, 5.0mL ethanol was added and heated on water bath for 2 minutes. A small piece of Magnesium ribbon was added to the alcoholic solution followed by 3 drops of concentrated HCl. Development of magenta red or orange colour indicated presence of flavanoids.
2. **Alkaline reagent Test:** Plant Extract and few drops of dilute NaOH gave Intense yellow colour. Addition of dilute HCl to yellow solution becomes colourless.

C) TEST FOR SAPONINS:

Frothing Test: 1.0mL extract was vigorously shaken with 10.0mL of distilled water in a test tube for 30 seconds and was left undisturbed for 20 minutes, Persistent froth indicated presence of saponins.

D) TEST FOR CARBOHYDRATES:

Molisch's test: To 1.0mL extract solution, α-napthol and concentrated H_2SO_4 were added. Purple colour indicated the presence of carbohydrates.

E) TEST FOR PHYTOSTEROL AND TRITERPENOIDS:

1. **Liebermann Burchardt's test:** To the small portion of each extract 5 drops of acetic anhydride was added on a cavity tile. Mass was mixed properly and 2 drops of concentrated H_2SO_4 was added. Formation of red, purple or green colour indicated presence of sterols and terpenoids.
2. **Salkowski's reaction:** To the small portion of each extract, 3.0mL of chloroform and 2.0mL of concentrated H_2SO_4were added and shake well. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence indicating presence of steroids.

F) TEST FOR TANNINS:

Reaction with lead acetate: To each dried extract, 2.0mL of 10% solution of lead acetate was added. Yellowish white precipitates indicated presence of tannins.

G) TEST FOR PHENOLIC COMPOUDS:

Test with $FeCl_3$: Each dried extract was dissolved in methanol and to this 2.0mL of freshly prepared $FeCl_3$ solution was added. Development of brownish green colour indicated presence of phenolic compounds.

H) TEST FOR GLYCOSIDES:

Legal's test: To the each extract, few drops of pyridine and sodium nitroprusside were added and was made alkaline with NaOH. A pink or red colour indicated presence of glycoside.

TABLE 2.1.2.3.1 *Anisomeles malabarica* R.B leaves

CONSTITUENTS	P	T	C	E	M	W
Alkaloids						
a) Dragendorff's test	-	-	-	-	+	-
b) Hager's test	+	+	-	+	+	+
c) Wagner's test	+	-	-	-	-	-
d) Mayer's test	-	-	-	-	+	+
Flavonoids						
a) Shinoda test	-	-	-	-	+	-
b) Alkaline reagent NaOH test	+	-	-	+	+	+
Saponins						
a) Foam test	-	-	-	+	+	+
Carbohydrates						
a) Molisch‟s test	-	-	-	-	-	-
Glycosides						
a) Legal‟s test	+	+	-	-	-	-
Phytosterols & Triterpenes						
a) Libermann Burchard test	-	-	-	-	-	-
b) Salkowski test	+	+	-	+	-	+
Phenolic						
a) Ferric chloride test	+	-	+	-	+	-
b) Bromine water test	-	-	-	+	+	-
Tannins						
a) Lead acetate test	-	-	-	-	-	-

(+) = indicates presence, (-) = indicates absence.

P= Petroleum ether 60-80° C, T= Toluene, C= Chloroform, E= Ethyl acetate, M= methanol, W= Water.

TABLE 2.1.2.3.2 *Anisomeles malabarica* R.B flowers

CONSTITUENTS	P	T	C	E	M	W
Alkaloids						
e) Dragendorff's test	+	-	-	-	+	-
f) Hager's test	-	+	+	-	+	+
g) Wagner's test	-	-	-	+	-	-
h) Mayer's test	-	-	-	-	-	+
Flavonoids						
c) Shinoda test	-	-	-	-	+	-
d) Alkaline reagent NaOH test	+	+	-	-	+	+
Saponins						
b) Foam test	+	-	+	-	+	+
Carbohydrates						
b) Molisch"s test	-	-	-	-	-	-
Glycosides						
b) Legal"s test	+	-	-	-	+	-
Phytosterols & Triterpenes						
c) Libermann Burchard test	+	-	-	-	-	-
d) Salkowski test	+	-	+	-	+	-
Phenolic						
c) Ferric chloride test	-	-	-	+	+	+
d) Bromine water test	-	-	-	-	-	-
Tannins						
b) Lead acetate test	-	-	-	-	-	-

(+) = indicates presence, (-) = indicates absence.

P= Petroleum ether 60-80° C, T= Toluene, C= Chloroform, E= Ethyl acetate, M= methanol, W= Water.

2.1.2.3 RESULTS AND DISCUSSION:

The phytoconstituents are known to play an important role in bioactivity of medicinal plants.

In phytoconstituents analysis the maximum number of class of compound were detected in methanol solvent followed by ethyl acetate and chloroform.

In qualitative phytochemical analysis, phenolics, flavonoids, and alkaloids class of compounds were present in high amount as compared to other phytoconstituents analyzed. The presence of alkaloids, phenolic compounds, flavonoids have been associated with various degrees of anti-inflammatory, antioxidant analgesic and antioxidant activities.

Therefore, the anti-inflammatory, and anti-oxidant effects observed which are associated with arthritis in this study may be due to the activity(s) of one or a combination of some of the classes of compounds present in *Anisomeles malabarica* R.BR plants.

2.1.2.4 REFERENCES:

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SECTION II
OPTIMIZATION OF EXTRACTION CONDITION

2.2. INTRODUCTION:

The optimization of extraction conditions plays an important role in selecting the solvent environment. These solvent extracts may possess the therapeutic value and determine the amount of compound of interest from the selected plants.

This chapter also include the volume of solvent and time taken for complete extraction.

2.2.1 SOLVENT OPTIMIZATION:

The successive solvent extraction was performed by subjecting the dry powdered and paste of the selected plants. Various solvents in order of increasing polarity were used.

PROCEDURE:

1. Approximately two gram each of plant sample were weighed and transferred to separate 10.0 cm^3 standard volumetric flasks.
2. 10.0 cm^3 of petroleum ether, ethyl acetate, chloroform and methanol were added separately to these flasks.
3. The contents of each flask were mixed and sonicated for 15 minutes with intermediate shaking.
4. Then these solvents were filtered through Whatman No.1 filter paper in separate pre-weighed dry beakers and each filtrate was evaporated to dryness on a water bath.
5. Each dried residue was then weighed and the percentage extractive values were calculated and tabulated for all solvents used.

2.2.1.1 OBSERVATIONS:

PLANTS NAME	% EXTRACTION OF SOLVENTS			
	Petroleum Ether	Chloroform	Ethyl Acetate	Methanol
Anisomeles malabarica R.BR Leaves	7.21	11.47	8.08	29.49
Anisomeles malabarica R.BR Flowers	5.17	10.25	10.13	36.10

2.2.1.2 RESULTS AND DISCUSSION:

The percentage extractive values from the different solvents selected for optimization was found to be high in methanol. The percentage methanolic extractive values of *Anisomeles malabarica* R.BR leaves and flowers were found to be 29.49 and 36.10 respectively.

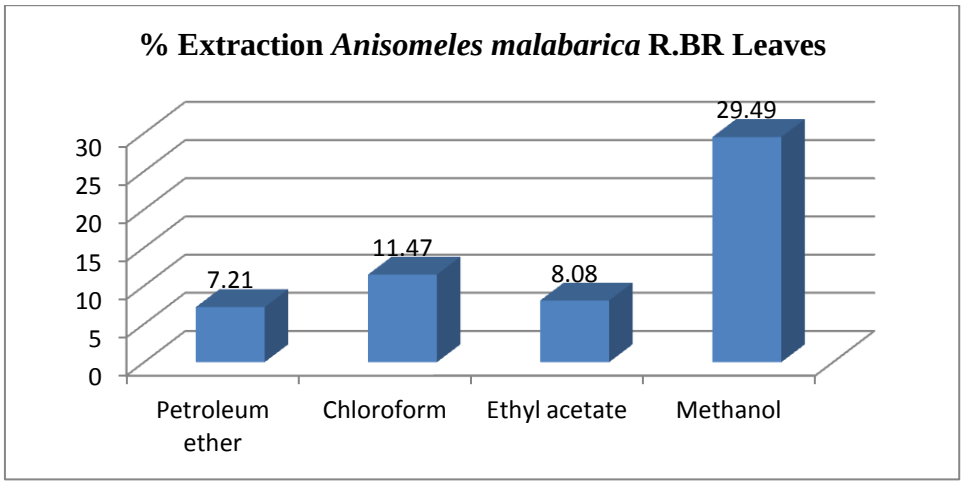

Figure 1: SOLVENT OPTIMIZATION:

***ANISOMELES MALABARICA* R.BR LEAVE**

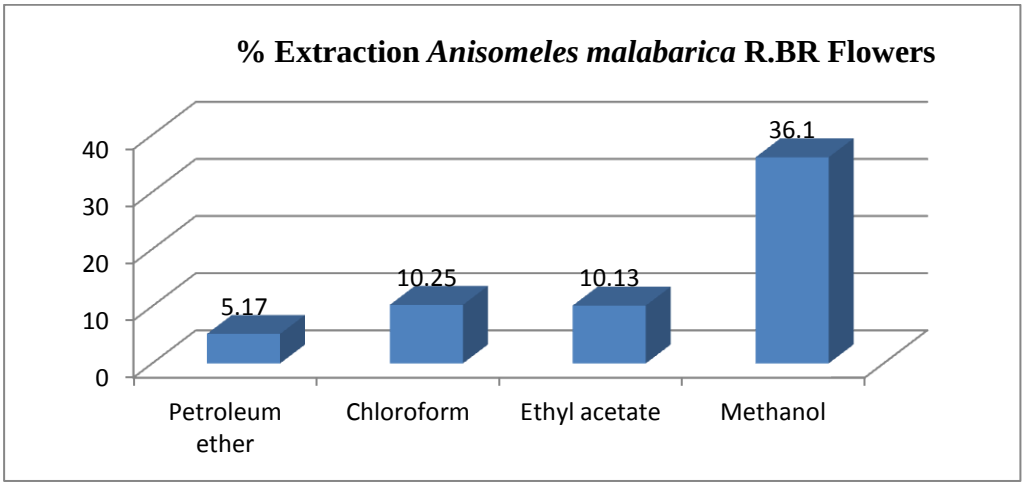

Figure 2: SOLVENT OPTIMIZATION:

***ANISOMELES MALABARICA* R.BR FLOWERS**

2.2.2 VOLUME OPTIMIZATION:

The volume optimization was performed by treating the dry powdered and paste of the selected plants with methanol. Increasing volumes of methanol from 10.0 cm^3 to 100.0 cm^3 were used.

PROCEDURE:

1. Approximately two gram each of the plant sample were weighed and transferred to different standard volumetric flasks. 10.0 cm^3 to 100.0 cm^3 of methanol were added separately to these flasks.
2. The contents of each flask were mixed and sonicated for 15 minutes with intermediate shaking.
3. Then the solvents were filtered through Whatman No.1 filter paper in separate pre-weighed dry beakers and each filtrate was evaporated to dryness on a water bath.
4. Each dried residue was then weighed and the percentage extractive values were calculated and tabulated.
5. From the percent extractive values, volume of methanol needed for extraction was optimised.

2.2.2.1 OBSEVATIONS:

PLANTS NAME	% EXTRACTION OF VOLUMES				
	10.0mL	25.0mL	50.0mL	75.0mL	100.0mL
Anisomeles malabarica R.BR Leaves	19.10	23.74	30.59	30.34	30.17
Anisomeles malabarica R.BR Flowers	11.37	15.64	36.10	35.93	36.05

2.2.2.2 RESULTS AND DISCUSSION:

It was found that the optimised volume of methanol solvent for extraction of about 2000 mg *Anisomeles malabarica* R.BR leaves and flowers were 50.0 cm^3 - 50.0 cm^3 respectively.

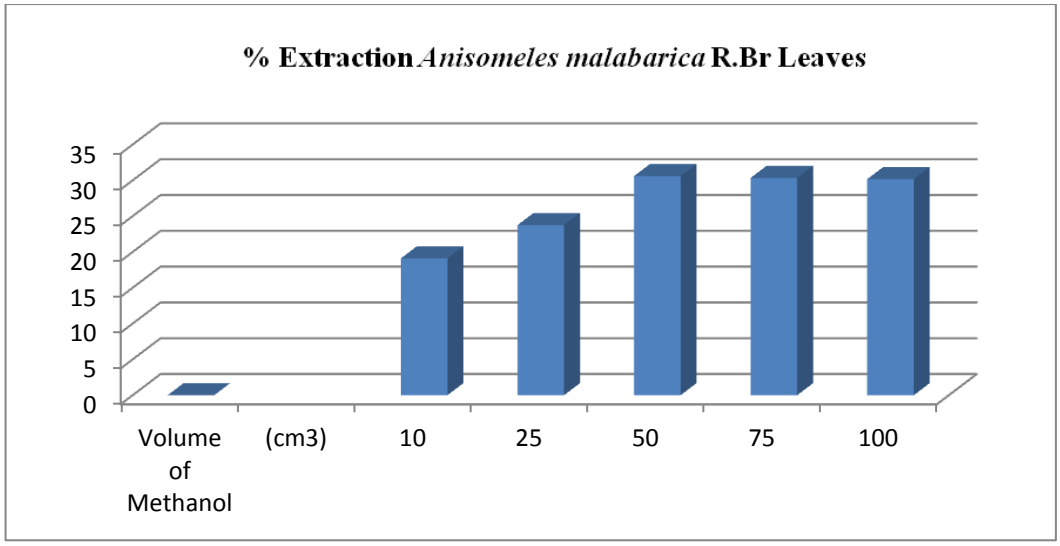

Figure 3: VOLUME OPTIMIZATION: *ANISOMELES MALABARICA* R.BR LEAVES

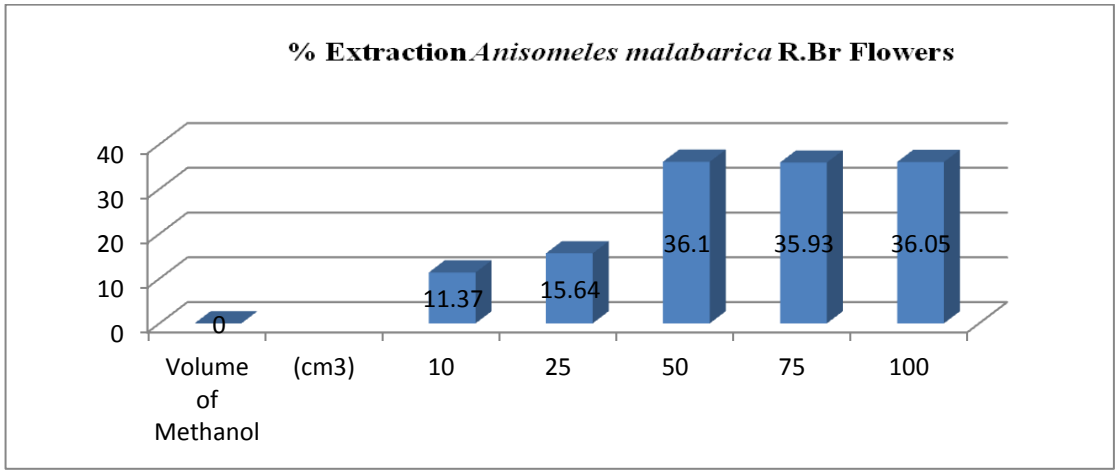

Figure 4: VOLUME OPTIMIZATION: *ANISOMELES MALABARICA* R.BR FLOWERS

2.2.3 TIME OPTIMIZATION:

PROCEDURE:

1. Approximately two gram each of the plant samples were weighed and transferred and the optimized volume of methanol were added to standard volumetric flasks.
2. The contents of each flask were mixed and subjected for a sonication for a time interval of 10 minutes to 60 minutes.
3. The contents of each flask were filtered through Whatman No.1 filter paper in separate pre-weighed dry beakers and methanol from each beaker was evaporated to dryness on a water bath.
4. Each dried residue was then weighed and the percentage extractive values were calculated and tabulated.
5. From the percent extractive values, optimised time of extraction were determined.

2.2.3.1 OBSERVATIONS:

PLANTS NAME	% EXTRACTION OF TIME				
	10.0 mins	20.0 mins	30.0 mins	40.0 mins	50.0 mins
Anisomeles malabarica R.BR Leaves	31.19	30.74	31.09	30.94	31.17
Anisomeles malabarica R.BR Flowers	18.57	27.64	36.45	35.73	36.13

2.2.3.2 RESULTS AND DISCUSSION:

It was observed that methanol percentage extractive values were remained constant after 10.0 minutes for leaves and 30.0 minutes for *Anisomeles malabarica* R.BR leaves and flowers were 10.0 minutes and 30.0 minutes respectively

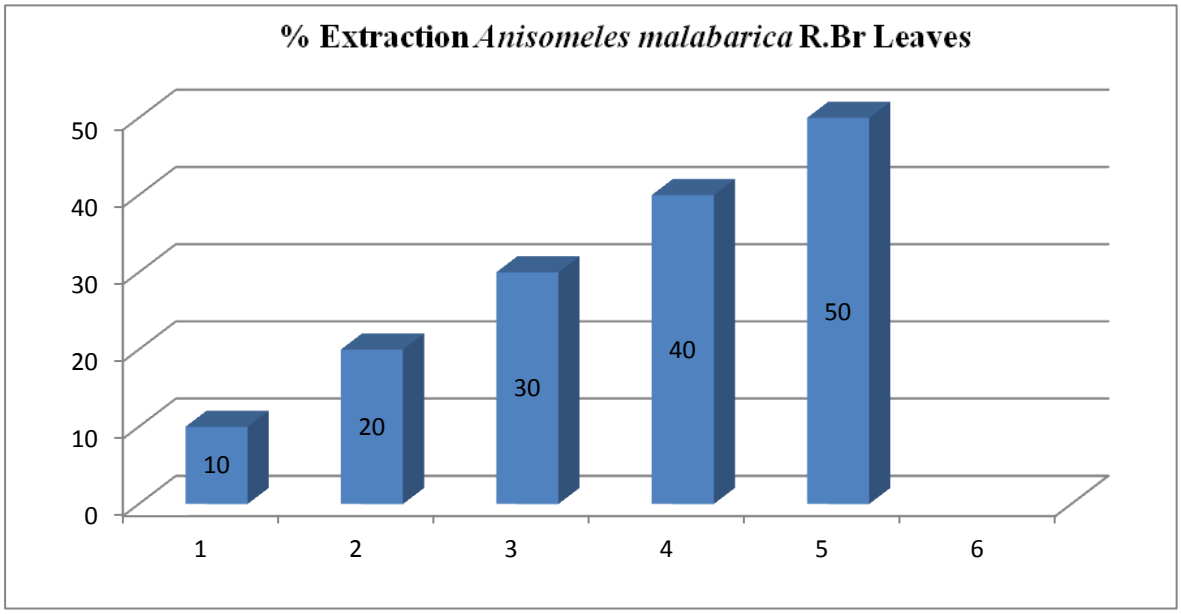

Figure 5: TIME OPTIMIZATION: *ANISOMELES MALABARICA* R.BR LEAVES

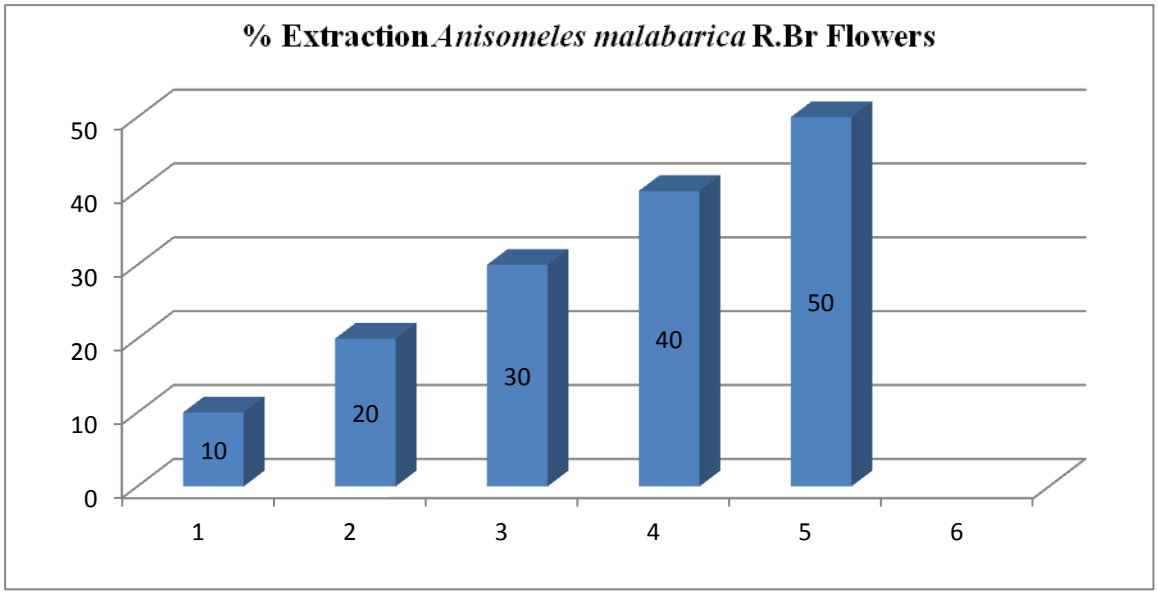

Figure 6: TIME OPTIMIZATION: *ANISOMELES MALABARICA* R.BR FLOWERS

2.2.4 RESULTS AND DISCUSSION:

The results obtained from the optimization of conditions are shown in the following table for *Anisomeles malabarica* R.Br leaves & flowers. These data can be used for the assessment of the quality of the drugs prepared from the selected plant.

It was found that maximum percent of the active phytochemicals were extracted in methanol solvent for the selected plant parts.

The volumes required for extraction were between 25.0mL to 50.0mL for the plant.

Time also plays an important role while preparing extracts, tincture, decoction etc for medicinal use. The time required for complete extraction was found to be 10.0 to 30.0 minutes for the selected plants.

The optimization was carried out for solvents, volume; time will help in analysis for further study of the selected plant *Anisomeles malabarica* R.BR.

Plants Name	**Part Used**	**Weight of Sample (g)**	**Optimized Solvent**	**Optimized Volume (cm^3)**	**Optimized Time (minutes)**
Anisomeles malabarica R.BR	Leaves	2.0	Methanol	50.0	10.0
	Flowers	2.0	Methanol	50.0	30.0

CHAPTER III
EXTRACTION
OF
ESSENTIAL
AND
FIXED OIL

SECTION I

EXTRACTION TECHNIQUES

3.1.1 OVERVIEW OF EXTRACTION TECHNIQUES:

Medicinal plants are the richest bio resource of drugs for traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Aromatic plants are a source of fragrances, flavours, cosmeceuticals, health beverages and chemical terpenes. Medicinal and aromatic plants (MAPs) are traded as such in bulk from many developing countries for further value addition in developed countries. The first step in the value addition of MAP bio resources is the production of herbal drug preparations i.e. extracts, using a variety of methods from simple traditional technologies to advanced extraction techniques.

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. The products so obtained from plants are relatively impure liquids, semisolids or powders intended only for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, semisolid extracts and powdered extracts. Such preparations popularly have been called galenicals, named after Galen, the second century Greek physician. The purpose of standardized extraction procedures for crude drugs are to achieve the therapeutically needed portion and to reduce the inert material by treatment with a selective solvent which can be use as a medicinal agent in the form of tinctures and fluid extracts. It may be further processed to be incorporated in any dosage form such as tablets or capsules, or it may be fractionated to isolate individual chemical entities. Thus, standardization of extraction procedures contributes significantly to the final quality of the herbal drug.

3.1.2 GENERAL METHODS OF EXTRACTION OF MADICINAL PLANTS:

Following are the general methods employed for medicinal plant extraction.

1. **Maceration:** In this process, the whole or coarsely powdered crude drug is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, pressed and clarified by filtration or decantation after standing.
2. **Infusion:** Fresh infusions are prepared by soaking the crude drug for a short period of time with cold or boiling water.
3. **Digestion:** This is a form of maceration in which gentle heat is used during the process of extraction. It is used when moderately elevated temperature is not objectionable.
4. **Decoction:** In this process, the crude drug is boiled in a specified volume of water for a defined time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat-stable constituents. This process is typically used in preparation of Ayurvedic extracts called "quath" or "kawath".
5. **Percolation:** In this method a liquid or solvent is slowly pass through the filtering medium where the crude drug is placed and it is allowed to stand for 4 hours in a well closed container. It is most frequently used in the preparation of tinctures and fluid extracts. A percolator a narrow, cone-shaped vessel open at both ends is generally used.
6. **Hot Continuous Extraction (Soxhlet):** In this method, the finely ground crude drug is placed in a porous bag or "thimble" made of strong filter paper, which is placed in chamber of the Soxhlet apparatus. The extracting solvent in flask is heated, and its vapours condense in condenser. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent.

7. **Ultrasound Extraction (Sonication):** The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitations and finally the active phytoconstituents get extracted.
8. **Supercritical Fluid Extraction (SFE):** The general goals of this method are to reduced use of organic solvents and increased sample output. Generally, cylindrical extraction vessels are used for SFE.

3.1.3 GENERAL METHODS OF EXTRACTION OF ESSENTIAL OIL FROM AROMATIC PLANTS:

Following are the general methods employed for aromatic plant extraction. The types of volatile isolates that are obtained commercially from aromatic plants are essential oils, concretes, absolutes, pomades and resinoids. Essential oils are isolated from plant material by distillation whereas other volatile isolates are obtained by solvent extraction.

ESSENTIAL OIL EXTRACTION PROCESS:

There are a lot of methods to extract the essential oils; the vast majority of true essential oils are produced by distillation. Common techniques used for the extraction of essential oils are;

- Hydro-distillation
- Hydro-diffusion
- Effleurage
- Cold pressing
- Steam distillation
- Solvent extraction
- Microwave Assisted Process (MAP)
- Carbon dioxide extraction

1. Hydro-distillation:

The technique involves distillation of water that is in direct contact with fresh or sometimes dried macerated plant materials. Plant material is grinded and weighed, then transferred into the Clevenger set up. Plant material is heated in two to three times its weight of water with direct steam. The distillation vessel is heated over heating mantle and the water vapour and oil are removed through a water cool condenser. An important factor to consider in water distillation is that the water present in the tank must always be enough to last throughout the distillation process; otherwise the plant material may overheat and char.

2. Hydro-diffusion:

Hydro-diffusion works on the diffusion principle of allowing steam to enter the top of the plant charge and diffuse through the charge by gravity. The process uses the principle of osmotic pressure to diffuse oil from the oil glands. In this method steam at atmospheric pressure (low-pressure steam <0-1 bar) is passed through the plant material from the top of the extraction chamber, thus resulting in the oils that retain the original aroma of the plants (Buchbauer, 2000). Hydro-diffusion is an efficient process because of its easy set up, especially regarding the processes of loading and unloading the plant material. The main advantage of this method is the higher yield of oil, less steam consumption, shorter distillation time and absence of hydrolysis, as the raw material does not come in contact with boiling water. However, because of the descending flow of steam and condensate, co-extraction of other non-volatile compounds (such as lipids, chlorophyll and fatty acids) and polar components makes the process complicated.

3. Enfleurage:

Enfleurage is the old fashioned method for extraction of essential oil. In this method essential oil is extracted with cold fat. This process is applicable to flowers such as jasmine or tuberose, that have low content of essential oil and so delicate that heating would destroy the blossoms before releasing the essential oils. Flower petals are placed on trays of odourless vegetable or animal fat which will absorb the flowers essential oil. Every day or every few hours after the vegetable or fat has absorbed as much essential oil as possible; the depleted petals are removed and replaced with fresh ones. This procedure continues until the fat or oil becomes saturated with the essential oil. This is called Enfleurage mixture. Addition of

alcohol helps to separate the essential oil from the fatty substances. The alcohol then evaporates leaving behind only the essential oil; hence enfleurage method is the best method when the source from the oil is to be extracted from flower or petals.

4. Cold pressing:

Cold pressing method is used to obtain citrus fruits oils such as lemon, lime, etc. The fruits to be extracted are rolled over a trough with sharp projections that penetrate the peels, this pierce the tiny pouches containing the essential oil. The whole fruit is pressed to squeeze the juice and is separated from the juice by centrifugation.

5. Solvent extraction:

This method employs the solvent for extraction of the oils from the oil bearing materials. Generally, non –polar solvents benzene, toluene, petroleum ether are used for extraction. The solvent enters the plant to dissolve the oil waxes then the solvent is removed by distillation under reduced pressure leaving behind substance containing resin (resinoid), or a combination of wax and essential oil (known as concrete) . The semisolid concretes are extracted with absolute ethanol. The second extract is cooled to precipitate the waxes and then filtered. This wax free alcoholic solution is distilled under reduced pressure to remove alcohol and finally the essential oil.

6. Microwave assisted process (MAP):

Microwave radiation interacts with dipoles of polar and polarisable materials. The coupled forces of electric and magnetic components change direction rapidly (2450 MHz). Polar molecules try to orient in the changing field direction and hence get heated. The polar water molecules in plant tissue interacts with the electric and magnetic components causing the cell walls to rupture and release the essential oil trapped in the extra cellular tissue of the plants. This technique reduces the time of extraction and a good yield of the essence is obtained.

7. Carbon dioxide extraction:

In this technique, plant material is placed in a high pressure vessel and carbon dioxide is passed through the vessel. The carbon dioxide turns into liquid and acts as a solvent to extract the essential oil from the plant material. When the pressure is decreased, the carbon dioxide returns to a gaseous state leaving no residue behind.

Qualities of essential oil extracted with any of the techniques described above depend on the chemical composition of the oil.

8. Steam distillation:

This is the most common and oldest method of extracting essential oils. In this technique, the desired plant (fresh or sometimes dried) is first placed into the vessel. Next steam is added and passed through the plant that contains the plants aromatic molecules or oils. Once the plant releases these aromatic molecules it travels within a closed system towards the water cooling device and where vapours get condensed into liquid state. A real advantage of satellite steam generation is that the amount of steam can be readily controlled. Because steam is generated in a satellite boiler, the plant material is heated no higher than 100° C and, consequently, it should not undergo thermal degradation. The main drawback to steam distillation is the much higher capital expenditure needed to build such a facility.

PRESENT RESEARCH WORK:

In the present study, Steam and Hydro-distillation methods were employed for essential oil extractions of the selected plant *Anisomeles malabarica* R.BR.

Hot continuous method of extraction i.e. Soxhlet extraction were used for the fixed oil extraction.

The results of the percent yield of oil extracted were discussed and tabulated.

3.1.4 EXTRACTION OF ESSENTIAL AND FIXED OIL FROM *ANISOMELES MALABARICA* R.BR. LEAVES AND FLOWERS

3.1.4.1 Extraction of Essential Oils from Leaves and Flowers Using Steam and Hydro Distillation Method:

PROCEDURE:

1. 250g of fresh leaves and flowers of *Anisomeles malabarica R.Br.* were chopped and placed in 1000mL of round bottom flask.
2. Heat is generated in a boiler made up of copper still and the distilling pot is infused with steam is allowed to pass the plant material which carries the Oil's vapour into the distilling head and then into the condenser, where the Oil and Water co-condense.
3. The distillation was carried out for 90 minutes.
4. The distillates were saturated with NaCl and extracted with n-Hexane.
5. The distillate was separated using separating funnel and solvent was evaporated using rota-vapour apparatus.
6. Anhydrous Sodium sulphate was added to dry organic phase.
7. The extraction was repeated in triplicates and the percentage content of essential oil obtained was calculated on the basis of material initially applied.
8. The oil extracted from the plant part was stored in a screw capped vials, under refrigeration.

Both leaves and flowers of the selected plant *Anisomeles malabarica* R.Br. were subjected to Steam and Hydro-distillation techniques (Figure 1 and 2) and the percentage yield result were tabulated (Refer Table 3.1.4.1).

Figure 1: Steam Distillation

Figure 2: Hydro-distillation using Clevenger Apparatus

OBSERVATIONS:

Table: 3.1.4.1

TECHNIQUE	PLANT NAME: *Anisomeles malabarica* R.BR	
	LEAVES	FLOWERS
STEAM DISTILLATION	0.013%	0.05%
HYDRO DISTILLATION	0.010%	0.065%

3.1.4.2 Extraction of Essential Oils from Leaves Using Hot Continues Extraction Technique (Soxhlet Extraction) Method:

PROCEDURE:

1. Dried leaves of *Anisomeles malabarica* R.BR were cleaned and powdered.
2. Fifty gram of powdered drug was then subjected to Soxhlet Extraction with 200mL of Petroleum Ether for about 6 hours till sufficient amount of oil was obtained (Figure 3).
3. At the end of extraction, the liquid extract was filtered and evaporated to complete dryness in a vacuum at 70 °C using a rota-vapour apparatus.
4. Finally, the dried extract was stored at 4 °C for further studies. The extraction was performed in triplicate and amount of fixed oil present were calculated and tabulated (Refer Table 3.1.4.2).

Figure 3: Soxhlet Extraction

Preparation of Fatty Acid Methyl Esters (FAMEs):

For preparation of ester derivatives of fixed oil AOAC Official method were employed.

PROCEDURE:

1. One gram of oil was taken in 250 ml round bottom flask, to which 15.0mL of 0.5M methanolic NaOH was added and subjected to reflux for 60 minutes.
2. 20.0mL of BF3- Methanol was then added from top of the condenser and then again boiled for 30 minutes.
3. 5.0mL of Petroleum Ether was then added through the condenser, heated for 5 minutes, heating was stopped.
4. Immediately 15.0mL of saturated NaCl was added. The mixture was shaken vigorously for a minute, additional NaCl was then added so that petroleum ether solution floats into the neck of flask.
5. FAMEs were collected by means of syringe and kept in closely tight glass vial in refrigerator [1].

OBSERVATIONS:

Table: 3.1.4.2

TECHNIQUE	**PLANT NAME: *Anisomeles malabarica* R.Br LEAVES**
SOXHLET EXTRACTION	1.16%

3.1.5 RESULTS AND DISCUSSION:

Two extraction techniques; Steam distillation and Hydro-distillation were employed for the extraction of essential oil from the plant *Anisomeles malabarica* R.BR. The investigation of the effect of percent yield obtained from these techniques shows that Hydro-distillation method is superior over steam distillation.

The percent oil yield obtained from Hydro-distillation method is comparatively high as compared to Steam distillation method (Refer Table 3.1.4.1). Therefore, Hydro-distillation is considered for the further study for carrying out the essential oil extraction.

For fixed oil extraction hot continuous extraction method i.e. Soxhlet Extraction method were used. The percentage amount yield of fixed oils present in the leaves shows high percent content of fixed oil in the selected plant *Anisomeles malabarica* R.BR (Refer Table 3.1.4.2).

The fixed oil was then subjected to their ester derivative formation for the analysis of its compositions using Gas chromatography mass spectrometry.

The results of chemical compositions of essential oils and fixed oils present in the both the plants were analysed by Gas chromatography mass spectrometry discussed in the preceding chapter of chromatography analysis.

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SECTION II

PHYSICO-CHEMICAL PARAMETER

ESSENTIAL OIL

3.2.1 INTRODUCTION:

Analysis plays an important role in assessment and preservation of quality and safety of essential oils obtained from plant used for multiple purposes by mankind since ancient times. The use of essential oils as food ingredients has a history dating back to ancient times. There are many examples of the use of citrus and other squeezed (manually or mechanically expressed) oils for sweets and desserts in ancient Egypt, Greece, and the Roman Empire. The rapid development of the fragrance and flavour industry in the nineteenth century was generally based on essential oils and related natural products. In recent days, aromatherapy associated with the concept of the holistic use of essential oils to promote health and well-being. There are virtually thousands of "aromatherapy "products in pharmacies, high street shops, supermarkets, hair salons, and beauty salons. The products are supposedly made with "essential oils" (which are usually perfumes) and include skin creams, hair shampoos, shower gels, moisturizers, bath salts, lotions, candles, as well as essential oils themselves.

Apart from the great interest in performing systematic studies on essential oils, there is also the necessity to trace adulterations, mainly in economically important essential oils. As can be observed with almost all commercially available products, market changes occur rapidly, affecting individual plants, or industrial processes. In general, market competition, along with the limited interest of consumers with regard to essential oil quality, may induce producers to adulterate their commodities by the addition of products of lower value. Different types of adulterations can be encountered: (a) the simple addition of natural and/or synthetic compounds, with the aim of generating oil characterized by specific quality values, such as density, optical rotation, residue percentage, ester value, and so on or (b) refined sophistications in the reconstitution and counterfeiting of commercially valuable oils.

As a result of diffused illegal practice in the production of essential oils, various international standard regulations have been introduced in which the characteristics of specific essential oils are described, and the botanical source and physicochemical requirements are reported. Moreover, guidelines for the analysis of essential oils are also available; for example, for the measurement of the refractive index (ISO 280, 1998) and optical rotation (ISO 592, 1998), as also for GC analysis using capillary columns [ISO chromatography (ISO 8432, 1987)] [1] .The French Standards

Association also develops norms and standard methods dedicated to the essential oil research field, with the aim of assessing quality in relation to specific physical, organoleptic, chemical, and chromatographic characteristics [2] .

3.2.2 CLASSICAL ANALYTICAL TECHNIQUES:

The earliest analytical methods applied in the investigation of an essential oil were commonly focused on quality aspects, concerning mainly two properties, namely identity and purity [4]. Physical behaviour of essential oils is designed by the natural plant components present in them. Physical parameters are widely quoted to evaluate purity and quality of essential oils. In trading of these oils, any impurity or adulteration, if practiced, can also be detected easily by matching the physical characteristics of essential oils with the already established required standards [5].

The following techniques are commonly applied to assess essential oil physical properties [3, 4]

1. **Specific gravity:**

The specific gravity of a substance is the ratio of density of the material to the density of water at a specified temperature. Specific Gravity can be expressed as

$$SG = \rho / \rho H2O$$

Where,

SG = specific gravity

ρ = density of fluid or substance (kg/m^3)

$\rho H2O$ = density of water (kg/m^3)

It is common to use the density of water at 4^0C (39^oF) as reference - at this point the density of water is at the highest - 1000 kg/m^3 or 62.4 lb/ft^3. Water is the standard for solids and liquids, while hydrogen is the standard for gases. The density of a liquid at a particular temperature is the mass (e.g. gram) of unit volume (e.g. 1.0mL) of the liquid. The density of solid indicates the weight of a substance held in a unit volume. Densities of liquids are generally measured either by weighing a definite volume of the liquid in a density bottle or pyknometer or by determining the buoyancy acting on a sinker immersed in a liquid.

2. **Optical rotation:**

Optical activity is determined by using a polarimeter, with the angle of rotation depending on a series of parameters, such as oil nature, the length of the column through which the light passes, the applied wavelength, and the temperature. The degree and direction of rotation are of great importance for purity assessments, since they are related to the structures and the concentration of chiral molecules in the sample. Each optically active substance has its own specific rotation, as defined in Biot's law:

$$[\alpha]^{T}_{\lambda} = \frac{\alpha}{c.l}$$

Where,

α is the optical rotation at a temperature T expressed in °C, l is the optical path length in dm, λ is the wavelength, and c is the concentration in g/100.0mL.

3. **Refractive index:**

This index is represented by the ratio of the sine of the angle of incidence (i) to the sine of the angle of refraction (e) of a beam of light passing from a less dense to a denser medium, such as from air to the essential oil.

$$\frac{Sine\ i}{sine\ e} = \frac{N}{n}$$

Where,

N and n are, respectively, the indices of the more and the less dense medium.

The Abbe-type refractometer, equipped with a monochromatic sodium light source, is recommended for routine essential oil analysis.

4. **Melting and Congealing points:**

The estimation of melting and congealing points, as well as the boiling range of essential oils, is also of great importance for identity and purity assessments. Melting point evaluations are a valuable modality to control essential oil purity, since a large number of molecules generally comprised in essential oils melt within a range of 0.5°C or, in the case of decomposition, over a narrow temperature range. On the other hand, the determination of the congealing point is usually applied in cases where the essential oil consists mainly of one molecule, such as the oil of cloves that contains about 90% of eugenol.

5. **Water solubility:**

The procedure applied for the purity assessment of essential oils is based on water solubility; the test, which reveals the presence of polar substances, such as alcohols, glycols and their esters, and glycerine acetates, is carried out as follows: the oil is added to a saturated solution of sodium chloride, which after homogenization is divided into two phases; the volume of the oil, which is the organic phase, should remain unaltered; volume reduction indicates the presence of water-soluble substances.

Following are the Classical methodologies have been widely applied to assess essential oil chemical properties [3, 4]

1. **Saponification value:**

Saponification value is defined as the amount of KOH in mg required in saponifying completely 1.0g of oil or fat. It is also a measure of the mean molecular weight of the fatty acids originally bound as triglycerides.

When fat is saponified by refluxing with a known excess of alcoholic potassium hydroxide solution, the triglycerides hydrolyze, while glycerol and soap are formed. The alkali consumed for this hydrolysis is a measure of the saponification value, which is determined by titrating the excess alkali with standard hydrochloric acid.

2. **Iodine number determination:**

Iodine value is a measure of level of unsaturation in fat. Iodine value is the amount of iodine in (g) absorbed per 100.0g of the oil or fat. The material is treated, in carbon tetrachloride medium, with a known excess of iodine monochloride solution in glacial acetic acid (Wijs solution). The excess of iodine monochloride is treated with potassium iodide and the liberated iodine estimated by titration with standard sodium thiosulphate solution.

3. **Acid value determination:**

Acid value is the amount of KOH in milligram, required to neutralize the free fatty acids present in 1.0g of the oil or fat. It is determined by directly titrating the material in an alcoholic medium with aqueous sodium or potassium hydroxide solution. Acid value is an important physicochemical property index of oil which is used to determine the quality, age, edibility and suitability of oil for industrial use such as paint [5]. This value is used to measure the extent of glycerides in the oil, which have been decomposed by lipase and other physical factors such as light and heat [6].

4. Thin Layer Chromatography:

Essential oils are also often analyzed by means of chromatographic methods. Planar chromatography may be referred to as a classical method for essential oil analysis, being well represented by thin-layer chromatography (TLC) and paper chromatography (PC). TLC is a fast and inexpensive method for identifying substances and testing the purity of compounds, being widely used as a preliminary technique providing valuable information for subsequent analyses. The R_f value obtained in TLC is characteristic for any given compound on the same stationary phase using the identical mobile phase. Hence, known R_f values can be compared to those of unknown substances to aid in their identification [7].

PRESENT RESEARCH WORK:

In present research work the essential oil obtained from the selected plant *Anisomeles malabarica* R.BR was subjected for both physical and chemical evaluation. The physical parameter includes the measurement of Colour, Odour, Specific gravity and Optical rotation. The chemical examination included the determination of Saponification value, Iodine number and Acid value.

3.2.3.1 PHYSICAL PARAMETERS:

1. **Colour Determination:** Colour of the respective oils was determined by physical observation in day light and under ultraviolet radiation of 254 and 366 nm using ultra violet chamber [8].
2. **Odour Determination:** Odour of the respective oils was determined by organoleptic evaluation following Evans [9].
3. **Specific Gravity:**

Procedure:

1. Specific gravity of the oil was determined with the help of a specific gravity tube using Pkynometer.
2. A Pkynometer tube was weighed and the weight was reported as W_1
3. The tube was then filled first with essential oil and again weighed; the weight was reported as W_2.
4. Distilled water is then added to the same tube and the weight was reported as W_3.
5. The specific gravity was calculated using the formula with respect to water.

$$\text{SPECIFIC GRAVITY} = \frac{\text{W2} - \text{W1}}{\text{W3} - \text{W1}}$$

Where,

W_1= Weight of empty tube

W_2= Weight of empty tube + Essential oil

W_3= Weight of empty tube + Distilled water

4. **Optical Rotation:**

Procedure:

1. 10.0mL Polari meter tube containing oil was placed in the cell of the instrument between polarizer and analyzer.
2. Care was taken in filling the tube to avoid the air bubble formation which could disturb the rotation of light.
3. Analysis was carried out using the Autopol IV automatic polarimeter Rudolph research analyzer.
4. The specific rotation was calculated and tabulated.

3.2.3.2 CHEMICAL PARAMETERS:

1. **Saponification Value:**

Procedure:

1. 0.5 g of each oil sample was weighted into a clean dried conical flask and 25.0mL of alcoholic potassium hydroxide was added.
2. A reflux condenser was attached to the flask and heated for an hour with periodic shaking.
3. The appearance of clear solution indicated the completion of saponification.
4. 1.0mL of 1 % phenolphthalein indicator was added and the hot excess alkali was titrated with 0.5 M hydrochloric acid until it reached the end point where it turned colourless.
5. A blank titration was carried out at the same time and under the same condition. The Saponification value was calculated and tabulated [12].

2. **Acid Value:**

Procedure:

1. 0.5g of oil was taken in a flask. 50.0mL of methylated spirit was added to the flask.
2. Shaken well and titrated against 0.1N KOH solution using phenolphthalein as indicator.
3. Alkali was added till a pink colour was established for a few seconds.
4. The acid value was then calculated and tabulated [12].

3. **Iodine Value:**

Procedure:

1. 0.1g of respective oil was weighed into a conical flask.
2. 10.0mL of chloroform and 25.0mL of the Wij's solution were added to the flask and the solution was kept in dark for 30 min at room temperature.
3. 10.0mL of 10 per cent potassium iodide solution with 100 ml of distilled water were added to the flask.
4. The resulting solution was titrated against 0.1N sodium thiosulphate, using starch as indicator till the end point where the blue black coloration becomes colourless.
5. A blank titration was carried out at the same time starting with 10.0mL of chloroform. Iodine value was then calculated and tabulated [13].

3.2.4 OBSERVATIONS:

TABLE 3.2.3 PHYSICOCHEMICAL CHARACTERISTICS

PARA METER	**AM EOs**		**AM FOs**
	Leaves	**Flowers**	**Leaves**
Colour	Pale yellow	Pale yellow	Colourless
Odour	Pleasant	Pleasant	Unpleasant
SG	0.766	0.771	0.841
OR	+10.0°	+10.8°	+50.5°
SAP mg KOH/g	102.47	100.17	151.67
IN g/100g	103.5	107.1	145
AN Mg KOH/g	1.71	1.79	1.58

AM = *Anisomeles malabarica* R.BR,

EOs = Essential Oil, FOs = Fixed Oil

SG = Specific Gravity, OR = Optical Rotation

SP = Saponification Value, IN = Iodine Number, AN = Acid Number

3.2.5 RESULTS AND DISCUSSION:

Studies of various physicochemical characteristics identify the practical importance and provide bases for suitability and utility of various oils of plants origin in daily life. Physicochemical properties of oil like colour, odour, specific gravity, refractive index, optical rotation, acid value, iodine value, saponification value etc indirectly tells about the quality of both essential and fixed oils.

In the present study essential oils and fixed oil obtained from the *Anisomeles malabarica* R.BR were evaluated for physicochemical characteristics **(Refer Table 3.2.3).** The essential oils of *Anisomeles malabarica* R.BR was found to be pale yellow in colour and fixed oil was colourless. Both the volatile had pleasant odour while fixed oil had unpleasant odour.

In the present study, fixed oils have high specific gravity values as compared to essential oils of both plants. The optical rotation was found to be high in fixed oils of both the plants.

The number of milligram of KOH, which are required to neutralize the free fatty acids present in one gram of oil. The acid number measures the amount of acids present in oil [14].Acid value is an indirect method for determination of free fatty acid of amount in oil samples and its edibility [14].Oil with low free fatty acids has more significant usage [15]. The Total acid number (TAN) values recorded in the present study. **(Refer Table 3.2.3).**

The number of milligram of KOH, which are required for the complete saponification of one gram of oil, is called saponification value. Saponification values determined and found to be very high in *Anisomeles malabarica* R.BR. All the oil samples are found in the range reported for plants (lower than the 188-196) but not useful in soup industry as these values are much lesser than the required value (±300) [16].

Iodine value is a Number of grams of iodine absorbed per 100 gram of fat or oil is known as Iodine value. It is the measure of unsaturation in oil. The iodine value is the indicative of fats and oils unsaturation. Fats and oils with higher unsaturation show high iodine value [17].The iodine valve observed for the oil samples in the present study were between 100-160g/100g oil. These values were found in the permissible range for semi-drying of oil (100-300). Recorded data for iodine number showed that all the oil samples have high unsaturated fatty acids contents [18].

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CHAPTER IV

SECTION I

GAS CHROMATOGRAPHY MASS SPECTROMETRY ANALYSIS

ANISOMELES MALABARICA R.BR

INRODUCTION TO ANALYTICAL METHODS:

The classification of essential oils depends on the resolution power of the analytical tools. The early method used for the characterization of essential oils was odour and colour. The earliest Classical analytical techniques for the systematic study of essential oils, such as specific gravity, optical activity, refractive index, or melting, congealing, and boiling points, and determination of iodine number are generally applied for the assessment of pure and major class of compounds.

Classical methods cannot be used as stand-alone methods and need to be combined with modern analytical techniques, especially GC and the hyphenated analytical tools, for the evaluation of essential oil genuineness.

Most of the modern analytical methods applied in the analysis of essential oils depend on chromatographic procedures, which facilitate components separation and identification.

The main purpose of any chromatographic separation is always the complete resolution of the compounds of interest, in the minimal time of period. To achieve this task, good knowledge of chromatographic theory, method optimization process, and development of innovative techniques must be used.

GAS CHROMATOGRAPHY (GC):

Gas chromatography has become the leading technique for the separation and analysis of thermally stable, volatile organic and inorganic compounds. Gas chromatographs are the most widely used analytical instruments in the world [1].

In gas chromatographic analysis, the compounds to be analyzed are vaporized and eluted by the mobile gas phase, the carrier gas, through the column. The analyte are separated on the basis of their relative vapor pressures and affinities for the stationary phase. The inert gaseous mobile phase does not interact with molecules of the analyte; its only function is to transport the analyte through the column.

The principal advantages of gas chromatography are [2]

(i) The technique has strong separation power and even quite complex mixtures can be resolved into constituents

(ii) Few micro litter samples are sufficient for analysis and high sensitivity

(iii) Non destructive, making possible on-line coupling e.g. to Mass spectrometer

(iv) Highly accurate quantitative analysis, typical RSD of 1-5%.

(v) Fast and sensitive, easily detecting ppm and often ppb

HYPHENATED TECHNIQUES:

Gas chromatography is often coupled with the selective techniques of spectroscopy, called hyphenated methods. This provides influential and practical tools to the chemist for identifying the components of complex mixtures.

Gas Chromatography - Mass Spectrometry (GC-MS) and Gas Chromatography - Infrared Spectroscopy - Mass Spectrometry (GC-IR-MS) are the modem analytical methods used for the separation and identification of components of essential oils.

Using these hyphenated techniques, identification of even trace components has become possible. The Fourier-Transform GC-IR, high resolution GC-MS and chemical ionization GC-MS are more powerful and selective characterization tools for the structure elucidation of components of oils.

When GC coupled with MS, the mass spectrometer, a universal detector for gas chromatography, convert the compounds into ions. At the same time the highly specific nature of a mass spectrum makes the mass spectrometer a very specific gas chromatographic detector. Gas chromatography is an ideal separator whereas mass spectrometry is excellent for identification. Gas chromatographic equipment can be directly interfaced with rapid scan mass-spectrometers of various types.

The main advantages of a mass spectrometer as a detector for gas chromatography are its increased sensitivity and its specificity in identifying unknown or confirming the presence of suspected compounds. GC-MS has been used for the identification of hundreds of components that are present in natural and biological systems. For example the characterization of the odour and flavor components of foods, identification of water pollutants, medical diagnosis based on breath components and studies of drug metabolites.

PRESENT RESEARCH WORK:

This section is further sub divided into two sections. In the present research work a gas chromatography- mass spectrometry method was developed for the selected plant; *Anisomeles malabarica* R.BR. The result obtained from the GC-MS for essential and fixed oil compositions of both the plant were tabulated and discussed.

4.1.1 EXPERIMENTAL: ESSENTIAL OIL OF LEAVES

4.1.1.1 Extraction of essential oil: *Anisomeles malabarica* R.BR Leaves

Around 250 g of fresh leaves of *Anisomeles malabarica* R.BR were cut into small pieces and submitted to hydro-distillation for 4 hours using a Clevenger-type apparatus. Standard method recommended by European Pharmacopoeia (1983) was followed. The oils obtained were kept in separating funnel for 12 hours. After separations the oil was then dried over anhydrous sodium sulphate. The experiment was conducted in triplicate.

4.1.1.2 Gas chromatography–mass spectrometry (GC/MS) analysis:

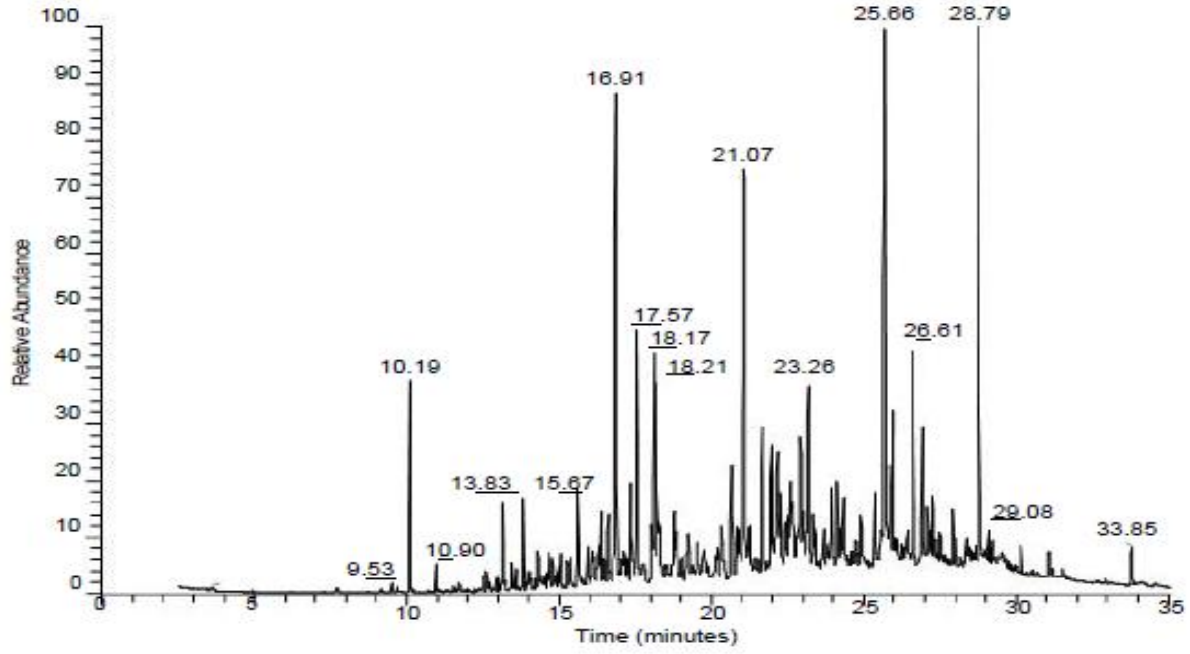
The essential oil was subjected to GC-MS analysis for phytochemical studies. GC-MS analysis of the sample was carried out using Shimadzu QP-2010 with non polar 60 M RTX 5MS Column. Helium was used as the carrier gas and the temperature programming was set with initial oven temperature at 40^0C and held for 3 min and the final temperature of the oven was 350^0C with rate at 10^0C/min. 2.0µL sample was injected with split less mode. Mass spectra was recorded over 35 - 550 amu range with electron impact ionization energy 70eV. The total running time for a sample was 40 minutes. Quantitative determinations were made by relating respective peak areas to TIC areas from the GC-MS.

4.1.1.3 Identification of Phytoconstituents:

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with spectrum of known component stored in NIST library. Quantitative determinations were made by relating respective peak areas to TIC areas from the GCMS. The name, molecular weight, retention time and peak area percentage of the test materials was ascertained.

4.1.1.4 Method in Brief: *Anisomeles malabarica* R.BR Leaves

GC Parameters	
Column	Elite 5MS non polar capillary column (30 m × 0.25 mm i.d., 0.25-µm film thickness)
Injector temperature	240^{o}C
Carrier gas	Helium 1.0mL/min
Oven temperature	Hold at 60^{o}C for 5 min Heat at 5^{o}C/min to 180^{o}C Hold at 180^{o}C for 5 min
Injection mode	Split ratio 1:50
Mass Parameters	
Source	Electron ionization
Ion energy	70 eV
Mass range scanned	40-550 a.m.u.
Source temperature	225°C
Transfer line temperature	250°C
Solvent delay	2.0 minutes



Essential Oil Mass Chromatogram: *Anisomeles malabarica* R.Br Leaves

TABLE 1

Chemical composition of essential oil Identified by GC-MS:

***Anisomeles malabarica* R.Br (Leaves)**

PEAK NO	COMPOUNDS IDENTIFIED	CAS NO.	RETENTION TIME (Minutes)	RELATIVE (%)
1	α-Thujane	208-912-2	9.53	13.7
2	α-Pinene	67762-73-6	10.90	0.57
3	Camphene	79-92-5	10.19	4.21
4	Cymene	99-87-6	13.83	1.55
5	Citral	141-27-5	15.67	9.08
6	Sabinene	3387-41-5	16.26	0.66
7	β-Pinene	76231-76-0	16.91	5.19
8	Linalyl acetate	115-95-7	17.57	2.10
9	Eucalyptus	470-82-6	18.17	3.16
10	Limonene	5989-27-5	18.21	4.27
11	Myrcene	123-35-3	19.51	0.59
12	Bornyl acetate	76-49-3	21.07	3.41
13	Nerol	106-25-2	23.26	2.40
14	Carveol	99-48-9	24.28	1.25
15	Borneol	507-70-0	24.81	0.98
16	α-Terpeneol	8000-41-7	25.66	7.47
17	Azulene	275-51-4	26.31	2.68
18	Eugenol	97-53-0	28.79	8.47
19	α-Terpenyl acetate	8007-35-0	29.08	10.42
20	β-Farnesene	18794-84-8	29.54	2.67
21	Germacrene-D	37839-63-7	30.13	0.72
22	Caryophyllene	6753-98-6	30.71	2.98
23	Bicyclogermacrene	24703-35-3	32.96	0.49
24	δ-Cadinene	483-76-1	33.85	9.03

TABLE 2

Biological Activity of Essential Oil Composition Identified:

***Anisomeles malabarica* R.Br (Leaves)**

PEAK NO	COMPOUNDS IDENTIFIED	BIOLOGICAL ACTIVITY**
1	α-Thujane	Antibacterial, Pesticide, Insecticide
2	α-Pinene	Allelochemic, Anti-inflammatory, Antibacterial, Antiseptic, Expectorant, Sedative, Perfumery
3	Camphene	Allelophatic, Antioxidant, Flavour, Expectorant, Pesticide,
4	Cymene	Antimicrobial, antinociceptive, anti-inflammatory
5	Citral	Allergenic, Anti-inflammatory, Antioxidant, Antiulcer, Antiviral, Expectorant, Fungicide
6	Sabinene	Antibacterial, Antiseptic, Antiulcer, Perfumery
7	β-Pinene	Allergenic, Anti-inflammatory, Antiseptic, Pesticide, Perfumery
8	Linalyl acetate	Anaesthetic, Antioxidant, Flavour, Sedative, Perfumery
9	Eucalyptus	Antiseptic, antibacterial,antitussive,anthelmintic,expectorant
10	Limonene	Allergenic, antiasthmatic, Antibacterial, Anticancer, Anti-inflammatory, Antiseptic
11	Myrcene	Allergenic, Analgesic, Antioxidant, Fungicide
12	Bornyl acetate	Antibacterial, Antiviral, Expectorant, Pesticide, Sedative
13	Nerol	Antibacterial, Antiseptic, Antiulcer, Sedative, Perfumery
14	Carveol	
15	Borneol	Analgesic, Antibacterial, Anti-inflammatory, Antibrochitic, Nematicide, Perfumery
16	α-Terpeneol	-
17	Azulene	Antibacterial , Pesticide, Anti-inflammatory, Antiseptic, Antiulcer
18	Eugenol	Allergenic, Antibacterial, Anti-inflammatory, Antiviral, Antiseptic, Antiulcer, Insecticide
19	α-Terpenyl acetate	-
20	β-Farnesene	-
21	Germacrene-D	Pesticide, Pheromone
22	Caryophyllene	Allergenic, Antibacterial, Anti-inflammatory, Antiulcer, Sedative, Perfumery
23	Bicyclogermacrene	-
24	δ-Cadinene	Antibacterial , Anticariogenic, Pesticide

BIOLOGICAL ACTIVITY**- Dr. Dukes Database

4.1.2 EXPERIMENTAL: ESSENTIAL OIL FLOWERS

4.1.2.1 Extraction of essential oil: *Anisomeles malabarica* R.Br Flowers

Around 250 g of fresh flowers of *Anisomeles malabarica* R.Br were cut into small pieces and submitted to hydro-distillation for 4 hours using a Clevenger-type apparatus. Standard method recommended by European Pharmacopoeia (1983) was followed. The oils obtained were kept in separating funnel for 12 hours. After separations the oil was then dried over anhydrous sodium sulphate. The experiment was conducted in triplicate.

4.1.2.2 Gas chromatography–mass spectrometry (GC/MS) analysis:

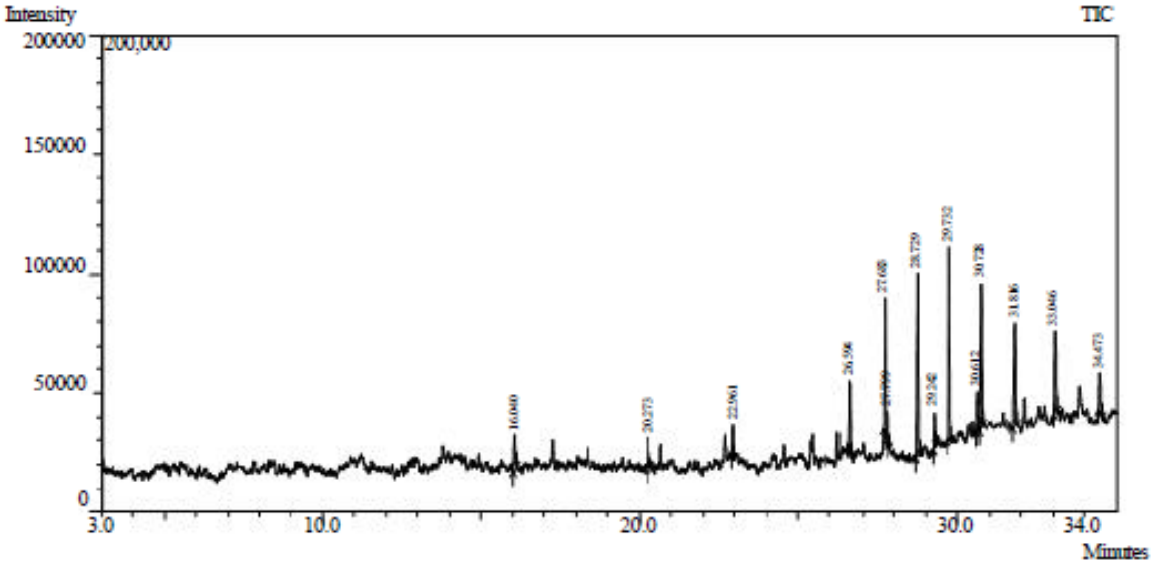
The essential oil was subjected to GC-MS analysis for phytochemical studies. GC-MS analysis of the sample was carried out using Shimadzu QP-2010 with non polar 60 M RTX 5MS Column. Helium was used as the carrier gas and the temperature programming was set with initial oven temperature at 40^0C and held for 3 min and the final temperature of the oven was 350^0C with rate at 10^0C/min. 2.0µL sample was injected with split less mode. Mass spectra was recorded over 35 - 550 amu range with electron impact ionization energy 70 eV. The total running time for a sample was 40 minutes. Quantitative determinations were made by relating respective peak areas to TIC areas from the GC-MS.

4.1.2.3 Identification of Phytoconstituents:

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with spectrum of known component stored in NIST library. Quantitative determinations were made by relating respective peak areas to TIC areas from the GCMS. The name, molecular weight, retention time and peak area percentage of the test materials was ascertained.

4.1.2.4 Method in Brief: *Anisomeles malabarica* R.BR Flowers

GC Parameters	
Column	Elite 5MS non polar capillary column (30 m × 0.25 mm i.d., 0.25-µm film thickness)
Injector temperature	240°C
Carrier gas	Helium 1.0mL/min
Oven temperature	Hold at 60°C for 5 min Heat at 5°C/min to 180°C Hold at 180°C for 5 min
Injection mode	Split ratio 1:50
Mass Parameters	
Source	Electron ionization
Ion energy	70 eV
Mass range scanned	40-550 a.m.u.
Source temperature	225°C
Transfer line temperature	250°C
Solvent delay	2.0 minutes



Essential Oil Mass Chromatogram: *Anisomeles malabarica* R.Br Flower

TABLE 1

Chemical composition of essential oil Identified by GC-MS:

***Anisomeles malabarica* R.Br (Flowers)**

PEAK NO	COMPOUNDS IDENTIFIED	CAS NO.	RETENTION TIME (Minutes)	RELATIVE (%)
1	Phenol, 2,4-bis(1,1-dimethylethyl)	96-76-4	16.040	3.38
2	1-Nonadecene	18435-45-5	20.273	2.06
3	1-Heptacosanol	2004-39-9	22.961	2.31
4	n-Dotriacontane	544-85-4	26.594	5.05
5	n-Tetracosane	646-31-1	27.683	10.39
6	Cyclodecasiloxane, eicosamethyl	18772-36-6	27.799	2.41
7	n-Tetracontane	4181-95-7	28.729	15.81
8	Tetracosamethyl-cyclododecasiloxane	18919-94-3	29.242	2.25
9	Tetrapentacontane	5856-66-6	29.732	16.76
10	Cyclononasiloxane, octadecamethyl-	556-71-8	30.612	3.02
11	n-Hexatriacontane	630-06-8	30.728	13.03
12	n-Tetratriacontane	14167-59-0	31.816	10.90
13	n-Tetracontane	4181-95-7	33.046	8.68
14	Tetracosane	646-31-1	34.473	3.94

TABLE 2

Biological Activity of Essential Oil Composition Identified:

***Anisomeles malabarica* R.Br (Flowers)**

PEAK NO	COMPOUNDS IDENTIFIED	BIOLOGICAL ACTIVITY**
1	Phenol, 2,4-bis(1,1-dimethylethyl)	Antioxidant, antibacterial
2	1-Nonadecene	Anti-fungal , Antioxidant
3	1-Heptacosanol	acid antibacterial and antifungal activity
4	n-Dotriacontane	Antimicrobial agent, hypercholesterolemic
5	n-Tetracosane	Antibacterial activity
6	Cyclodecasiloxane, eicosamethyl	Antioxidant, antibacterial
7	n-Tetracontane	Antibacterial activity
8	Tetracosamethyl-cyclododecasiloxane	Antioxidant, antibacterial
9	Tetrapentacontane	Antibacterial activity
10	Cyclononasiloxane, octadecamethyl-	Antioxidant, antibacterial
11	n-Hexatriacontane	Antibacterial and Antifungal activity
12	n-Tetratriacontane	Antibacterial activity
13	n-Tetracontane	Antibacterial activity
14	Tetracosane	Antibacterial activity

BIOLOGICAL ACTIVITY**- Dr. Dukes Database

4.1.3 EXPERIMENTAL: FIXED OIL OF FLOWERS

4.1.3.1 Extraction of Fixed Oil: *Anisomeles malabarica* R.Br Flowers

Fifty gram of powdered aerial plant part was subjected to Soxhlet Extraction with 200.0mL of Petroleum Ether for about 6 hours till sufficient amount of oil was obtained. At the end of extraction, the liquid extract was filtered and evaporated to complete dryness in a vacuum at 70 °C using a Rotavapor apparatus. The extraction was performed in triplicate.

4.1.3.2 Preparation of Fatty Acid Methyl Esters (FAMEs):

One gram of oil was taken in 250 ml round bottom flask, to which 15.0mL of 0.5M methanolic NaOH was added and subjected to reflux for 60 minutes. 20.0mL of BF3- Methanol was then added from top of the condenser and then again boiled for 30 minutes. 5.0mL of Petroleum Ether was then added through the condenser, heated for 5 minutes, heating was stopped. Immediately 15.0mL of saturated NaCl was added. The mixture was shaken vigorously for a minute, additional NaCl was then added so that petroleum ether solution floats into the neck of flask. FAMEs were collected by means of syringe. This oil (as the methyl esters of the fatty acids) was analyzed by GC-MS.

4.1.3.3 Gas chromatography–mass spectrometry (GC/MS) analysis:

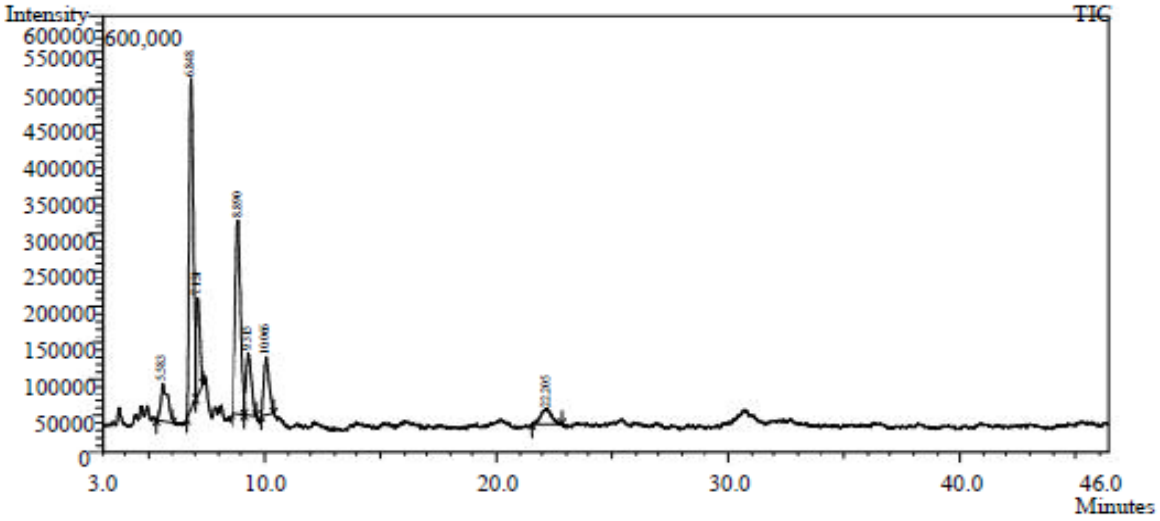
The Fatty esters of fixed oil were subjected to GC-MS analysis for phytochemical studies. GC-MS analysis of the sample was carried out using Shimadzu QP-2010 system equipped with a polar capillary SGE BX-70 column (30 m \ 0.25 mm) and helium as the carrier gas. The oven temperature was kept at 130^0C for 1 min, programmed to 185^0C at a rate of 5^0C/min and kept at 185^0C for 2 min, then programmed to 220^0C at a rate of 15^0C/min and kept at 220^0C for 3 min. The injection volume was 0.1μL in the split mode.

4.1.3.4 Identification of Phytoconstituents:

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST). The mass spectrum of the unknown component was compared with spectrum of known component stored in NIST library. Quantitative determinations were made by relating respective peak areas to TIC areas from the GCMS.

4.1.3.5 Method in Brief: *Anisomeles malabarica* R.Br Flowers

GC Parameters	
Column	Capillary SGE BX-70 column (30 m \ 0.25 mm)
Injector temperature	250^{o}C
Carrier gas	Helium 1.0mL/min
Oven temperature	Hold at 130^{o}C for 1 min Heat at 5^{o}C/min to 185^{o}C Hold at 185^{o}C for 2 min Heat at 15^{0}C/min to 220^{0}C Hold at 220^{0}C for 3 min
Injection volume	0.1µL split mode
Mass Parameters	
Source	Electron ionization
Ion energy	70 eV
Mass range scanned	35-550 a.m.u.
Source temperature	150°C
Transfer line temperature	200°C
Solvent delay	2.0 minutes



Fixed Oil Mass Chromatogram: *Anisomeles malabarica* R.Br Flower

TABLE 1

Chemical Composition of Fixed Oil Identified by GC-MS:

***Anisomeles malabarica* R.Br (Flowers)**

PEAK NO	COMPOUNDS IDENTIFIED	CAS NO.	RETENTION TIME (Minutes)	RELATIVE (%)
1	1-Heneicosanol	15594-90-8	5.583	6.71
2	Hexadecanoic acid	112-39-0	6.848	35.47
3	Delta -Undecalactone	104-67-6	7.131	9.19
4	Oleic Acid	112-62-9	8.890	27.87
5	Octadecanoic acid	112-63-0	9.315	8.74
6	Oleic Acid	112-62-9	10.066	7.95
7	Diisooctyl phthalate	27554-26-3	22.205	4.07

TABLE 2

Biological Activity of Fixed Oil Composition Identified:

***Anisomeles malabarica* R.Br (Flowers)**

PEAK NO	COMPOUNDS IDENTIFIED	BIOLOGICAL ACTIVITY**
1	1-Heneicosanol	Antimicrobial and antioxidant
2	Hexadecanoic acid (Palmitic acid)	Antioxidant, hypocholesterolemic, nematicidal, pesticidal, hemolytic, antiandrogenic
3	Delta -Undecalactone	Antioxidant
4	Oleic Acid	Anti-inflammatory
5	Octadecanoic acid (Steric acid)	Hypocholesterolemic
6	Oleic Acid	Anti-inflammatory
7	Diisooctyl phthalate	Antibacterial

BIOLOGICAL ACTIVITY**- Dr. Dukes Database

4.1.4 RESULTS AND DISCUSSION:

In Gas Chromatography Mass Spectrometry analysis total 24 components were present in the essential oil of *Anisomeles malabarica* R.Br leaves whereas 14 components were found in flowers.

Total 7 components was identified from the fixed oil in flower extracts of *Anisomeles malabarica* R.Br

The components identified by NIST library and their biological activity were identified from the Dr. Duke's Database.

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SECTION II

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

INTRODUCTION:

HPTLC stands for High Performance Thin Layer Chromatography. High performance thin layer chromatography (HPTLC) is a sophisticated instrumental technique based on the full capabilities of thin layer chromatography. The advantages of automation, scanning, full optimization, selective detection principle, minimum sample preparation, hyphenation, etc. enable it to be a powerful analytical tool for chromatographic information of complex mixtures of inorganic, organic, and bio-molecules.

Thin-layer chromatography, among various chromatographic techniques, score high over other chromatographic techniques as it is a valuable tool for reliable identification providing chromatographic fingerprints.

The feature that distinguishes TLC from other physical and chemical methods of separation is that two mutually immiscible phases are brought in to contact while one phase is stationary and the other mobile. A sample is loaded on the stationary phase and is carried by the mobile phase. Species in the sample undergo repeated interaction between the mobile and stationary phase. When both phases are properly selected, the sample components are gradually separated into bands or zones [1].

The attractive features of TLC are low-cost analysis of samples requiring minimal sample clean up and allows a reduction in the number of sample preparation steps. TLC is also preferred for the analysis of substances with poor detection characteristics requiring post-chromatographic treatment for detection.

It is used for the identification of drugs and toxic substances in biological fluids, unacceptable residue levels, maintaining a safe water supply by monitoring natural and drinking water sources for crop projecting agents used in modern agriculture, and confirmation of label claims for content of pharmaceutical products. It remains one of the main methods for class fractionation, speciation and flavour potential of plant materials. HPTLC technique is reported to be useful for identification of morphological and geographical variations in terms of chemical markers from various medicinally important plants [2]. It is frequently selected as the method of choice to study the metabolism and fate of radiolabel compounds in the body and environment.

ADVANTAGES OF HPTLC:

1. HPTLC allows fast, inexpensive method of analysis in the laboratory as well as in field.
2. The modern HPTLC technique, combined with automated sample application and densitometric scanning, is sensitive and completely reliable, suitable for use in qualitative and quantitative analysis.
3. HPTLC is a valuable tool for reliable identification because it can provide chromatographic fingerprints that can be visualized and stored as electronic images.
4. Special advantages of HPTLC include high sample throughput and low cost per analysis; multiple samples and standards can be separated simultaneously.
5. The sample preparation requirements are often minimal because the stationary phase is disposable.

PRESENT RESEARCH WORK:

In the present study a new methods were developed with some modification for the selected plant *Anisomeles malabarica* R.BR.

Simultaneous methods were developed and validated for the presence of Gallic acid and Quercetin from the aerial part of *Anisomeles malabarica* R.BR.

4.2.1 EXPERIMENTAL:

Simultaneous High Performance Thin Layer Chromatographic determination of Gallic acid and Quercetin from aerial Part of *Anisomeles malabarica* R.BR

Method in brief:

In the present research work, a sensitive and accurate, High Performance Thin Layer Chromatographic (HPTLC) method for simultaneous determination of Gallic acid and Quercetin in *Anisomeles malabarica* R.BR has been developed and validated. The analytes separations were achieved on aluminium plates precoated with silica gel $60F_{254}$. HPTLC plates with Toluene: Ethyl acetate: Formic Acid (4.5 cm^3: 3.0cm^3: 0.5cm^3) v/v/v as mobile phase. The development distance was 80 mm. Detection and quantification was performed by densitometry, with a deuterium lamp, at 366 nm. The response of Gallic acid and Quercetin was linear in the concentration range 0.6 to 1.2µg per band and 0.2 to 1.5µg per band respectively. The validated method was used for quantitative analysis of Gallic acid and Quercetin in *Anisomeles malabarica* R.BR plant and can be used for routine quality-control analysis of aerial powder of *Anisomeles malabarica* R.BR.

Literature survey:

Column chromatography of *Anisomeles malabarica* R.BR has been reported in literature, using Toluene: Ethyl acetate: Formic acid (5:4:1) as a mobile phase for the separation of quercetin from leaf powder of *Anisomeles malabarica* R.BR [1].

An HPTLC method has been reported for the quantitative determination of Gallic acid and Quercetin from Polyherbal formulation. In this method, the mobile phase comprising of Toluene: Ethyl acetate: Formic acid (5:4:1) was used and detection and quantitation were carried out at 366 nm [2].

An HPTLC method has also been reported for the quantitative determination of Quercetin from Cuscuta *reflexa* Roxb. In this method, the mobile phase comprising of Toluene: Ethyl acetate: Formic acid in the volume ratio of 5.0:2.0:0.5 was used and detection and quantitation of Quercetin was carried out at 366 nm [3].

However HPTLC method for simultaneous quantitation of Gallic acid and Quercetin has not been reported in literature for the selected plant.

Densitometric HPTLC has been widely used for the phytochemical evaluation of the herbal drugs, due to its simplicity and minimum sample clean up required. Hence densitometric HPTLC method has been developed in the present research work for simultaneous qauntitation of Gallic acid and Quercetin from methanol extract of aerial powder of *Anisomeles malabarica* R.BR.

4.2.1.1 Experimental conditions:

Reagents:

Analytical grade Toluene (purity 99.5%), Formic acid (purity 99.0%), Ethyl acetate (purity 99.9%), Petroleum ether (purity 99.5 %) were obtained from Qualigens Fine Chemicals, Mumbai, India.

Standards:

Reference standard Gallic acid (purity 95.0%) and Quercetin (purity 98.0%) were procured from Sigma-Aldrich Chemie GmbH (Aldrich Division, Steinbeim, Germany).

Plant material

The dried aerial powder of *Anisomeles malabarica* R.BR was used for the analysis.

Preparation of Stock Standard Solution:

Stock solution of 10.0mg/mL of Gallic acid and Quercetin were prepared in methanol. The contents were sonicated for 5 minutes and then diluted with methanol to give a solution of Gallic acid and Quercetin 1000µg/mL.

Table 1

Standard	Weight of Standard (mg)	Diluted standard stock solution (mL)	Concentration of standard stock solution (µg/mL)
Gallic acid	10.0	10.0	1000
Quercetin	10.0	10.0	1000

From the stock solution a series of solutions were prepared in the range of 0.6-1.2µg per band for Gallic acid and 0.5-1.4µg per band for Quercetin.

Table 2

	Standard			
	Gallic acid		Quercetin	
Serial no.	Volume µL	Concentration (µg/band)	Volume µL	Concentration (µg/band)
1	5.0	0.50	5.0	0.50
2	6.0	0.60	6.0	0.60
3	7.0	0.70	7.0	0.70
4	8.0	0.80	8.0	0.80
5	9.0	0.90	9.0	0.90
6	10.0	1.00	10.0	1.00
7	11.0	1.10	11.0	1.10
8	12.0	1.20	12.0	1.20
9	13.0	1.30	13.0	1.30
10	14.0	1.40	14.0	1.40

Sample preparation:

About 1000 mg of aerial powder of *Anisomeles malabarica* R.BR was accurately weighed and sonicated with 10.0 cm^3 of methanol for 15 minutes. The extract was filtered through a Whatman no. 1 filter paper, pore size 11.0µm, the contents were then evaporated to dryness and final volume was adjusted to 5.0 cm^3 with methanol in a volumetric flask.

Preparation of mobile phase:

The mobile phase was prepared by mixing toluene, ethyl acetate and formic acid in volume ratio of 4.5: 3.0: 0.5 and then sonicated for 10 minutes.

Instrumentation:

Aluminium backed HPTLC Silica gel 60 F_{254} (Cat No. 1.0.5554) plates having thickness of 200 µm (E. Merck, Germany) were used for the present analysis. The samples and standard solutions were applied as sharp bands by means of CAMAG (Muttenz, Switzerland) Automatic TLC Sampler 4(ATS 4),equipped with 25-µL Hamilton syringe. The development was carried out in a CAMAG glass twin trough chamber (20 x 10 cm and 10 x 10 cm). Densitometric scanning was performed with CAMAG TLC Scanner 3, using win CATS software, version 1.4.4 in absorbance /reflectance mode, using deuterium lamp at the wavelength of maximum absorption of Gallic acid and Quercetin, at λ= 366 nm, slit size, 6.0 x 0.45 mm.

The photo documentation of the plates was done using CAMAG TLC Visualiser.

Table 3

Optimised chromatographic conditions used for quantification of Gallic acid and Quercetin from aerial Part of *Anisomeles malabarica* R.BR

Parameters	**Description**
Stationary phase	Aluminium backed HPTLC Silica gel 60 F_{254} (Cat No. 1.0.5554) (E. Merck, Germany), 200µm thickness.
Mobile phase	Toluene : Ethyl acetate : Formic Acid ($4.5\ cm^3$: $3.0cm^3$: $0.5cm^3$) v/v/v.
Sample applicator	CAMAG Automatic TLC Sampler 4(ATS4), equipped with 25µL –Hamilton syringe.
Speed of application	Methanol-150nL/second.
Band length	8 mm.
Development chamber	CAMAG glass twin trough chamber (20 x 10 cm and 10 x 10 cm).
Chamber saturation	15 minutes with filter paper.
Development distance	60 mm from lower edge of plate.
Densitometer Scanner	CAMAG TLC Scanner 3 with win CATS software, version 1.4.4.
Wavelength of detection(λ)	366 nm.
Radiation Source	Deuterium

4.2.1.2 Selection of chromatographic conditions:

Mode of separation:

In the present research work, normal mode of separation was used, where stationary phase was comparatively more polar than mobile phase.

Stationary phase:

Separations can be performed using modified, non-modified and impregnated stationary phases due to a difference in chemical properties between the sorbent material and the compound of the sample to be separated. The unmodified stationary phases include silica, alumina, starch, kieselguhr, silicates, controlled–porosity glass, cellulose, starch, gypsum, polyamides and chitin. For TLC and HPTLC, the most frequently used stationary phase is silica. TLC/HPTLC layers are available on glass plates, aluminium sheets and polyester sheets. Although glass sheets should be preferred because of their durability, there are some advantages that make aluminium sheets an attractive choice. These advantages include a much lower weight and possibility of being easily cut into any desired choice. Aluminium backed TLC plate has a torsional strength that is almost as good as a glass plate. The most common pore sizes used in TLC are 40 A°, 60 A°, 80 A° and 100 A°, with silica gel 60 A° being far most popular and versatile of the group. Pore size affects selectivity and hence can be used to good effect in altering the migration rates and resolution of sample components. The plates have large surface area, particle diameter between 5 µm to 15 µm and have uniform film thickness. Hence, in the present research work, TLC silica gel 60 F_{254} plates were used.

Mobile phase:

In the present work, as the stationary phase was polar, the optimisation of mobile phase was started with a non-polar solvent like toluene, n- hexane and the combinations of different non polar, mid polar and polar solvents.

Development and drying:

Ascending mode of chromatographic development was chosen for present work. The plates were developed in a CAMAG glass twin trough chamber (20 x 10 cm and 10 x 10 cm) lined with filter paper and was previously saturated with mobile phase vapours for 20 minutes. The development was carried up to a distance of 60 mm. They were then dried with ambient air for 10 minutes.

Detection and quantification:

8.0µL of the working standard solution of Gallic acid and Quercetin corresponds to 0.8µg per band was applied to the same 5 x 10 cm TLC plate. The plate was then developed and scanned under optimised chromatographic conditions. (Table 4.2.2.3) The Gallic acid and Quercetin showed maximum absorbance at 366 nm. Therefore, 366 nm was selected for the detection and quantification of Gallic acid and Quercetin from the aerial powder of *Anisomeles malabarica* R.BR. This wavelength matches with the methods reported for the quantification of Gallic acid and Quercetin from different plants.

Densitometric scanning:

The plates were scanned using CAMAG TLC Scanner 3, with win CATS software**,** version 1.4.4, in absorbance/reflectance mode, using tungsten lamp at the wavelength of maximum absorption of Gallic acid and Quercetin at λ = 366nm , slit size 6.0 x 0.45 mm.

4.2.1.3 METHOD VALIDATION:

Validation is a systemic approach to identifying, measuring, evaluating, documenting, and re-evaluating all the critical steps responsible before establishing the validity of the method. The developed method is validated in terms of linearity, precision, limit of detection (LOD), limit of quantification, accuracy, robustness, and ruggedness.

The parameters that were considered for the validation of the HPTLC method for the quantification of Gallic acid and Quercetin from aerial powder of *Anisomeles malabarica* R.BR were,

1. Linearity and range
   a) LDR
   b) LWR
2. Limit of detection (LOD) and limit of Quantification (LOQ)
3. Precision
   a) Repeatability
   b) Intermediate Precision
4. Robustness
5. Accuracy
6. System suitability
7. Specificity
8. Solution Stability

1. Linearity and range:

A) Linear Dynamic Range (LDR):

This experiment was carried out to demonstrate the range over which the response of the detector is linear with respect to concentration of Gallic acid and Quercetin.

A series of working standard solutions of 0.5 to 1.4µg per band and 0.5 to 1.4µg per band were used for the determination of linear dynamic range for Gallic acid and Quercetin respectively. The bands were applied at a distance of 8.0 mm from bottom and plate was developed and scanned.

The densitograms were obtained for each applied concentration of Gallic acid and Quercetin and the peak areas of Gallic acid and Quercetin were noted for each applied concentration of Gallic acid and Quercetin. The values of peak areas of Gallic acid and Quercetin for each applied concentration of Gallic acid and Quercetin are represented in Table 4.

Table 4

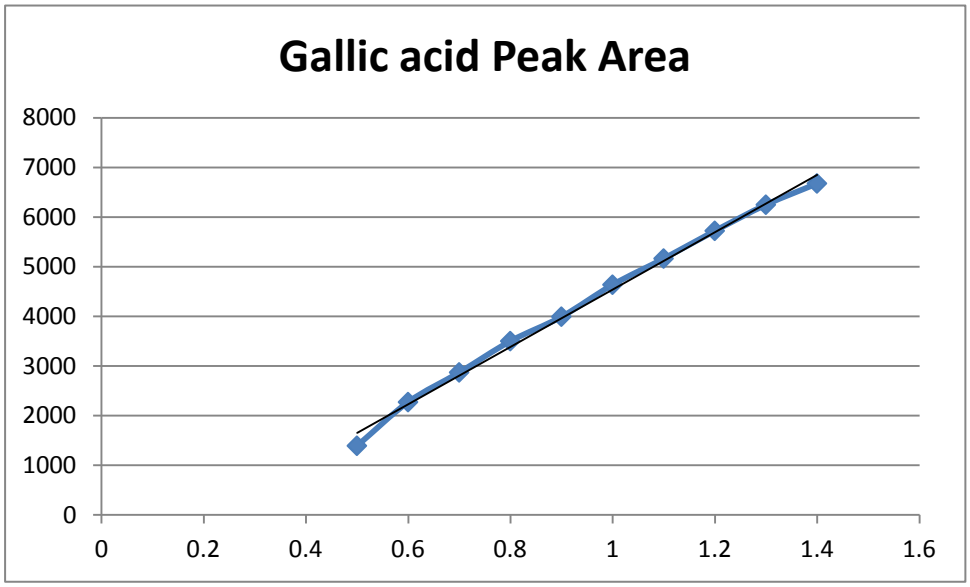
Linear dynamic range of Gallic acid and Quercetin

	Standard				
	Gallic acid			Quercetin	
Observation no.	**Concentration µg/band**	**Peak Area**		**Concentration µg/band**	**Peak Area**
1	0.50	1392.5		0.50	909.3
2	0.60	2273.0		0.60	1328.0
3	0.70	2871.1		0.70	1935.6
4	0.80	3501.2		0.80	2719.5
5	0.90	3993.3		0.90	3299.4
6	1.00	4637.4		1.00	4229.1
7	1.10	5164.6		1.10	4947.8
8	1.20	5723.0		1.20	5699.4
9	1.30	6249.0		1.30	6417.9
10	1.40	6677.0		1.40	7035.7

A graph of peak area values of Gallic acid and Quercetin (Y-axis) against the corresponding applied concentration of Gallic acid and Quercetin (X- axis) was plotted and is shown in Figure 1 and 2.

Figure 1

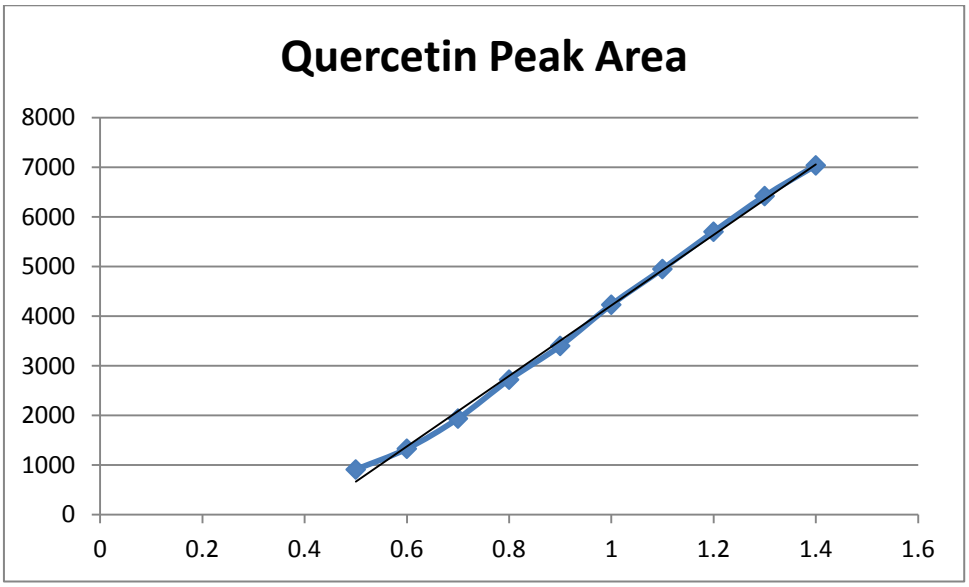
Linear dynamic range of Gallic acid



The graph shows that the response of Gallic acid is linear in the concentration range of 0.6µg per band to 1.3µg per band.

Figure 2

Linear dynamic range of Quercetin



The graph shows that the response of Quercetin is linear in the concentration range of 0.7µg per band to 1.4µg per band.

B) Linear Working Range (LWR):

Working standard solutions of Gallic acid and Quercetin in the concentration range of 0.6 to 1.3µg per band and 0.7 to 1.4µg per band respectively were applied in triplicate, to three different plates and developed and scanned using the optimized conditions described above. The densitograms were then acquired and the peak areas were recorded for each concentration of Gallic acid and Quercetin.

The values of mean peak areas, standard deviation (S.D.) and percent relative standard deviation (% R.S.D.) for each of its applied concentration were calculated. The results are represented in Table 5 and Table 6.

Table 5

Linear working range of Gallic acid

Obs. No	Concentration of GA (µg/band)	Peak Area of GA			Mean Peak Area	S.D	%R.S.D
1	0.60	2273.5	2275.0	2274.8	2274.43	0.8144	0.036
2	0.70	2871.1	2873.4	2872.8	2872.43	1.1930	0.042
3	0.80	3501.2	3501.9	3502.2	3501.77	0.5132	0.015
4	0.90	3993.3	3993.5	3995.1	3993.97	0.9866	0.025
5	1.00	4637.4	4636.0	4638.7	4637.37	1.3503	0.029
6	1.10	5164.6	5165.5	5163.1	5164.4	1.2124	0.023
7	1.20	5723.0	5724.1	5724.8	5723.97	0.9073	0.016
8	1.30	6249.0	6248.2	6250.2	6249.13	1.006	0.016

Figure 3

Mean Peak Areas of Gallic acid against Concentration of Gallic acid

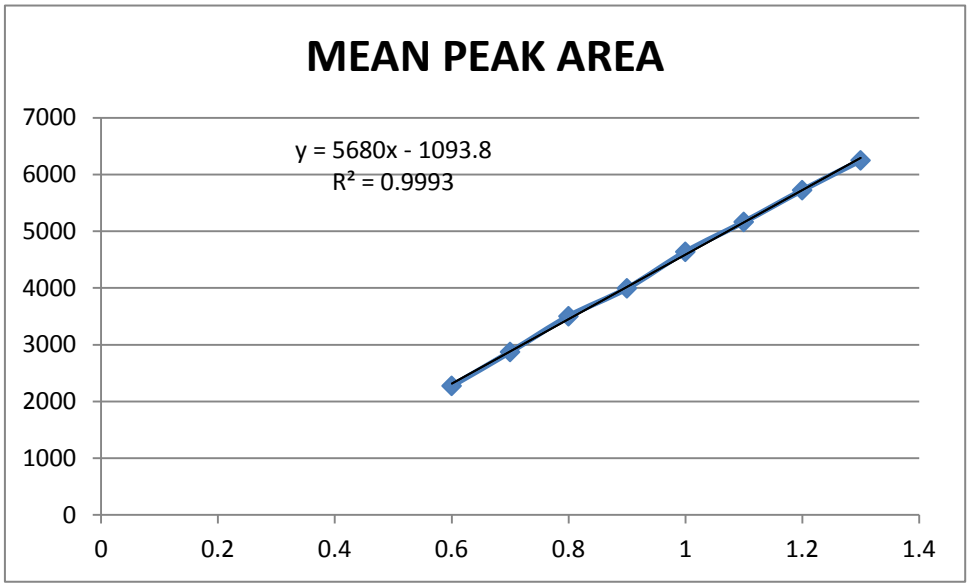


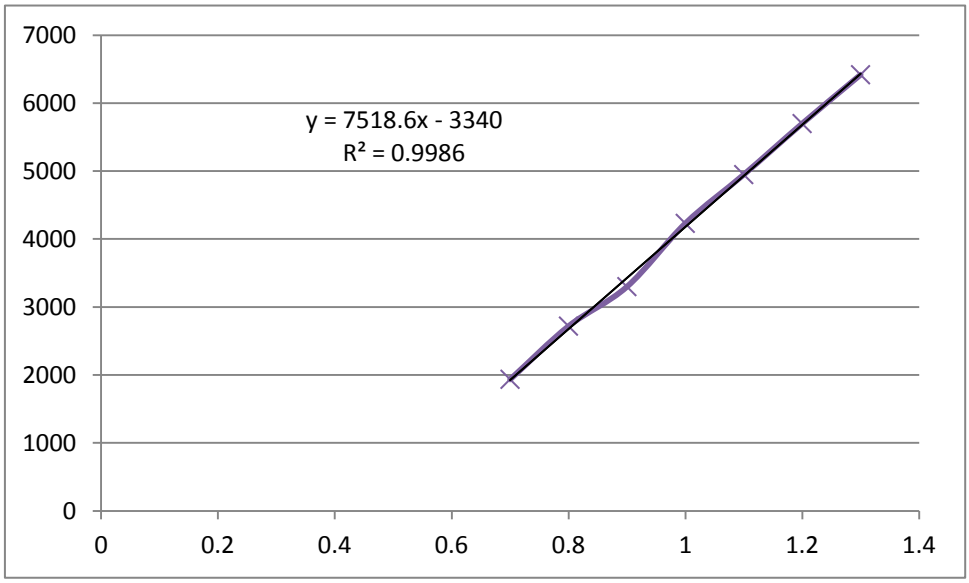
Table 4.2.2.6

Linear working range of Quercetin

Obs. No	Concentration of QC (µg/band)	Peak Area of QC			Mean Peak Area	S.D	%R.S.D
1	0.70	1935.6	1936.1	1937.0	1936.23	0.709	0.0366
2	0.80	2719.5	2719.3	2718.4	2719.07	0.586	0.0216
3	0.90	3299.4	3299.9	3300.1	3299.80	0.360	0.0109
4	1.00	4229.1	4231.0	4232.1	4230.73	1.518	0.0360
5	1.10	4947.8	4948.6	4948.2	4948.20	0.400	0.0080
6	1.20	5699.4	5698.4	5698.3	5698.70	0.608	0.0107
7	1.30	6417.9	6418.1	6417.2	6417.73	0.473	0.0074

Figure 4

Mean Peak Areas of Quercetin against Concentration of Quercetin



From the above graph, it is observed that Gallic acid and Quercetin a linear response in the concentration range of 0.6 to 1.30 and 0.7 to 1.3µg per band respectively. The results of linearity experiment were subjected to regression analysis.

Regression analysis:

The regression analysis of the calibration data was carried out to determine the relationship between the dependent variable (peak area of Gallic acid and Quercetin) and independent variable (concentration of Gallic acid and Quercetin).The regression equation is: $y = mx + c$

Where,

y = Mean peak area of Gallic acid and Quercetin

m = slope of the regression line.

x= Concentration of Gallic acid and Quercetin (µg/band)

c = Intercept on y – axis.

The values of correlation co-efficient, intercept and slope were determined from the graph of mean peak area of Gallic acid and Quercetin (Y–axis) against corresponding applied concentration of Gallic acid and Quercetin (X–axis).

The regression equation for Gallic acid was found to be, y = 5680x -1093. This indicates that 99.99% (correlation coefficient (r) × 100) of the variation in the response is explained by the variation in concentration of Gallic acid.

The regression equation for Quercetin was found to be, y = 7518x - 3340. This indicates that 99.80% (correlation coefficient (r) × 100) of the variation in the response is explained by the variation in concentration of Quercetin.

The results of the regression analysis for Gallic acid and Quercetin are given in Table 7

Table 7

Regression data for Gallic acid and Quercetin

Standard	Slope(m)	Intercept(c)	Correlation coefficient(r)
Gallic acid	5680	-1093	0.999
Quercetin	7518	-3340	0.998

3. Limit of detection (LOD) and Limit of Quantification (LOQ):

The values of LOD and LOQ were determined at a signal to noise ratio of 3:1 and 10:1 respectively. The values of **Limit of detection** (LOD) and **Limit of Quantification** (LOQ) obtained for Gallic acid were 0.6 and 0.7µg/band and Quercetin were 0.7 and 0.8µg/band respectively.

Table 8

LOD AND LOQ for Gallic acid and Quercetin

Standard	LOD µg/band	LOQ µg/band
Gallic acid	0.6	0.7
Quercetin	0.7	0.8

4. Precision:

The variability of the method was studied by carrying out for repeatability and intermediate precision.

A. Repeatability

The repeatability experiment was carried out in the same laboratory, on the same day, by analyzing six replicates of sample solutions of aerial powder of *Anisomeles malabarica* R.BR.

About 1000 mg of aerial powder of *Anisomeles malabarica* R.BR was accurately weighed six times. Each sample solution was prepared and extracted with methanol as described earlier. Each sample solution was applied in six replicates on the same day, as 8 mm bands to a 20 x 10 cm TLC plate, and analysed using optimized chromatographic conditions (Table 5.4).

The values of peak areas of Gallic acid and Quercetin obtained in the aerial sample solutions were recorded. The values of mean peak areas, standard deviation (S.D.) and percent relative standard deviation (% R.S.D.) for Gallic acid and Quercetin are given in Table 9.

Table 9

Repeatability of Gallic acid and Quercetin from aerial powder of *Anisomeles malabarica* R.BR

Obs. No	Weight of aerial powder (mg)	Peak area of Gallic acid from aerial powder	Peak area of Quercetin from aerial powder
1	1002	2984	3562
2	1006	2985	3715
3	1002	2986	3565
4	1004	2985	3562
5	1007	2987	3598
6	1001	2988	3565
Mean	**1003.7**	**2985.8**	**3594.5**
S.D.	**2.422**	**1.472**	**4.274**
% R.S.D.	**0.241**	**0.05**	**0.131**

As the values of percent relative standard deviation (% R.S.D.) for the peak areas of Gallic acid and Quercetin in aerial powder solutions are below 2, it indicates that the method is precise for performing the analysis.

B. Intermediate precision:

Intermediate precision was carried out like repeatability by analyzing six replicates of sample solutions of aerial powder of *Anisomeles malabarica* R.BR. but it was carried out on three successive days. About 1000 mg each of aerial powder of *Anisomeles malabarica* R.BR was accurately weighed six times on each day. The leaf powder sample solutions were prepared and extracted as described earlier. The test solutions aerial powder of *Anisomeles malabarica* R.BR were prepared as described earlier. The values of peak areas of Gallic acid and Quercetin obtained in aerial powder solutions were noted on each day. The values of mean peak areas, standard deviation (S.D.) and percent relative standard deviation (% R.S.D.) for Gallic acid and Quercetin were calculated. The results of intermediate precision of three successive days are represented in Table 10, 11 and Table 12 respectively.

Table 10

Intermediate precision of Gallic acid and Quercetin from aerial powder of *Anisomeles malabarica* R.BR

(Day 1)

Obs. No	Weight of aerial powder (mg)	Peak area of GA from aerial powder	Peak area of QC from aerial powder
1	1001	2985	3565
2	1005	2987	3563
3	1003	2986	3564
4	1005	2986	3565
5	1001	2988	3566
6	1005	2987	3567
Mean	**1003.3**	**2986.5**	**3565**
S.D.	**1.96**	**1.049**	**1.414**
%R.S.D.	**0.196**	**0.035**	**0.04**

Table 11

Intermediate precision of Gallic acid and Quercetin from aerial powder of *Anisomeles malabarica* R.BR

(Day 2)

Obs. No	Weight of aerial powder (mg)	Peak area of GA from aerial powder	Peak area of QC from aerial powder
1	1001	2986	3567
2	1005	2985	3565
3	1003	2986	3566
4	1007	2986	3567
5	1007	2987	3568
6	1003	2985	3569
Mean	**1004.3**	**2985.7**	**3567**
S.D.	**2.422**	**0.816**	**1.414**
%R.S.D.	**0.241**	**0.03**	**0.04**

Table 12

Intermediate precision of Gallic acid and Quercetin from aerial powder of *Anisomeles malabarica* R.BR

(Day 3)

Obs. No	Weight of aerial powder (mg)	Peak area of GA from aerial powder	Peak area of QC from aerial powder
1	1007	2990	3560
2	1001	2985	3562
3	1005	2988	3562
4	1003	2989	3565
5	1002	2987	3565
6	1006	2988	3566
Mean	**1004.0**	**2987.8**	**3563.3**
S.D.	**2.366**	**1.722**	**2.34**
%R.S.D.	**0.236**	**0.056**	**0.066**

As the values of percent relative standard deviation (% R.S.D.) for peak areas of Gallic acid and Quercetin present in aerial powder of *Anisomeles malabarica* R.BR for six replicates on three successive days are below 2, it indicates that the method is precise for performing the analysis.

5. System suitability:

The system suitability test was carried out to confirm that system used is adequate to carry out analysis and gives reproducible results. System suitability was studied by applying Gallic acid and Quercetin standard solution (0.7µg per band and 0.8µg per band) six times to same TLC plate and analysed using the optimized conditions.

The densitograms were obtained for each applied concentration of Gallic acid and Quercetin. The peak areas were noted for each applied concentration of Gallic acid and Quercetin.

The parameters used to determine the system suitability were reproducibility of the peak areas and retention factors (R_f) of Gallic acid and Quercetin for replicate analysis. The values of mean peak area, standard deviation (S.D.) and percent relative standard deviation (% R.S.D.) obtained for Gallic acid and Quercetin are given in Table 13.

Table 13

System suitability

Obs. No	Concentration of GA (µg/band)	Retention factor (R_F)	Peak area of GA	Concentration of QC (µg/band)	Retention factor (R_F)	Peak area of QC
1	0.70	0.24	3502.9	0.80	0.41	2046
2	0.70	0.26	3502.2	0.80	0.40	2055
3	0.70	0.24	3502.6	0.80	0.41	2035
4	0.70	0.25	3502.1	0.80	0.40	2045
5	0.70	0.25	3501.8	0.80	0.41	2058
6	0.70	0.24	3502.9	0.80	0.42	2060
	Mean	**0.247**	**3502.42**	**Mean**	**0.408**	**2049.83**
	S.D.	**0.008**	**0.453**	**S.D.**	**0.008**	**9.537**
	% R.S.D.	**1.31**	**0.013**	**% R.S.D.**	**1.84**	**0.465**

As the values of percent relative standard deviation for both peak areas and retention factors (R_f) of Gallic acid and Quercetin are below 2, it indicates that the system is suitable and can be used for routine chromatographic analysis.

6. Specificity:

Specificity of the method was determined by changing the chromatographic conditions like use of HPTLC Silica gel 40 F_{254} plate during analysis, and change in the solvents of mobile phase.

The mobile phase comprising of combinations of non-polar, mid polar and polar solvents were used for separation. However it was observed that a good resolution of the components of the sample was obtained when the mobile phase comprised of Toluene: Methanol: Formic Acid (4.5 cm^3: 3.0cm^3: 0.5cm^3) v/v/v was used for the analysis.

7. Solution stability:

Solution stability was determined using freshly prepared standard solution of Gallic acid and Quercetin. It was kept at room temperature for 24 hours. 8.0µL of Gallic acid and Quercetin standard solution, corresponding to 0.80µg per band was applied at the end of 4, 8, 12 and 24 hours respectively and used for analysis.

The densitograms were recorded for each applied concentration of Gallic acid and Quercetin. The peak area values of Gallic acid and Quercetin were noted. The values of mean peak areas, standard deviation (S.D.) and percent relative standard deviation (% R.S.D.) were calculated. The results of solution stability for Gallic acid and Quercetin given in Table 14.

Table 14

Stability studies

Obs. No	Time (Hours)	Concentration of GA (µg/band)	Peak area of GA	Concentration of QC (µg/band)	Peak area of QC
1	0	0.80	3502.9	0.80	2720.3
2	4	0.80	3502.2	0.80	2719.7
3	8	0.80	3502.6	0.80	2719.4
4	12	0.80	3502.1	0.80	2720.6
5	24	0.80	3501.8	0.80	2720.1
		Mean	**3502.32**	**Mean**	**2720.02**
		S.D.	**0.4324**	**S.D.**	**0.4764**
		% R.S.D.	**0.0123**	**% R.S.D.**	**0.0175**

As the values of percent relative standard deviation (% R.S.D.) for the peak areas of Gallic acid and Quercetin are below 2, it indicates that the solution is stable at room temperature (28 ± 2^0C) for a minimum time period of 24 hours under normal laboratory conditions.

4.2.1.4 ASSAY APPLICATION OF THE VALIDATED HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF GALLIC ACID AND QUERCETIN FROM AERIAL POWDER OF *ANISOMELES MALABARICA* R.BR

The validated HPTLC method was used for the quantitation of Gallic acid and Quercetin the aerial powder of *Anisomeles malabarica* R.BR. The aerial powder of *Anisomeles malabarica* R.BR were prepared by weighing accurately about 1000 mg of aerial powder in seven separate 10.0 cm^3 standard volumetric flasks.10.0 cm^3 of methanol was added to each flask. The contents of each flask were mixed and each flask was then sonicated for 15 minutes with intermediate shaking. The contents of each flask were then filtered through Whatman No.1 filter paper and the filtrate of each sample solution was evaporated to dryness. Final volume was adjusted to 5.0 cm^3 with methanol in a volumetric flask.

For the assay of Gallic acid and Quercetin from the Aerial powder solutions, volumes of 10.0µL of test solutions (prepared as described above) were applied seven times each as 8 mm bands, to the same 20 x 10 cm TLC plate, by means of CAMAG Automatic TLC Sampler 4(ATS4). The plate was developed and scanned under optimised chromatographic conditions (Table 4.2.2.3).

The identities of the peaks of Gallic acid and Quercetin from the aerial powder solutions were confirmed by comparing the chromatograms of sample solutions with that of standard Gallic acid and Quercetin. The retention factor of Gallic acid standard and Gallic acid present in aerial powder of *Anisomeles malabarica* R.BR was found to be 0.24 and the retention factor of Quercetin standard and Quercetin present in aerial powder of *Anisomeles malabarica* R.BR was found to be 0.40.

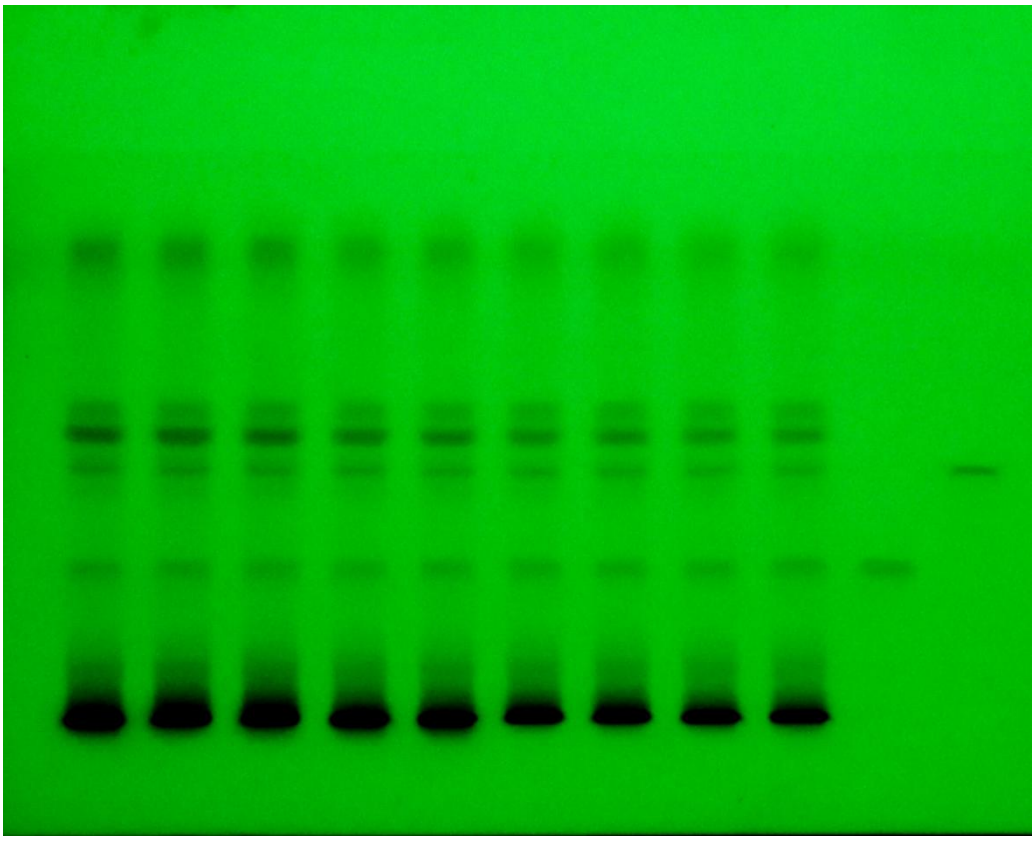
The amounts of Gallic acid and Quercetin present in each sample solution and the values of standard deviation (S.D.), percent relative standard deviation (% R.S.D.) were calculated. The results of assay experiment of Gallic acid and Quercetin present in aerial powder solutions are given in Table 15.

Typical HPTLC chromatogram of Gallic acid and Quercetin present in the methanolic extract of aerial powder of *Anisomeles malabarica* R.BR shown in Figure 6.

Figure 5 shows a TLC plate shows separation of standard Gallic acid and Quercetin and Gallic acid and Quercetin present in methanolic extract of aerial powder of *Anisomeles malabarica* R.BR.

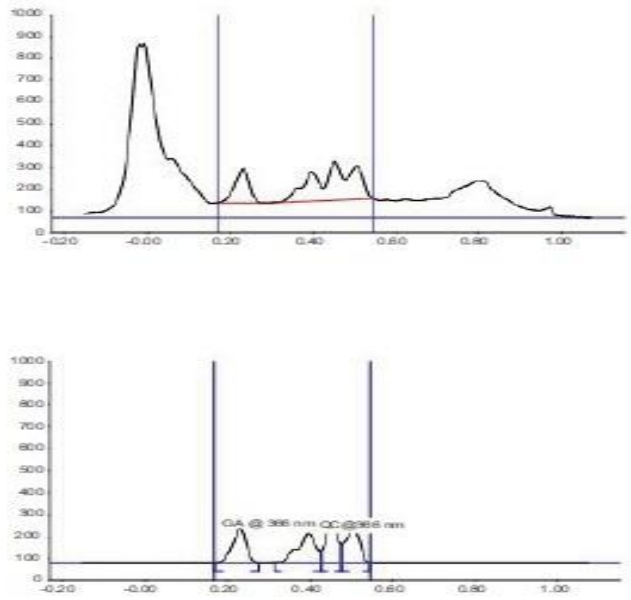
Figure 5

TLC plate showing separation of standards Gallic acid and Quercetin and L Gallic acid and Quercetin present in methanol extract aerial powder of *Anisomeles malabarica* R.BR

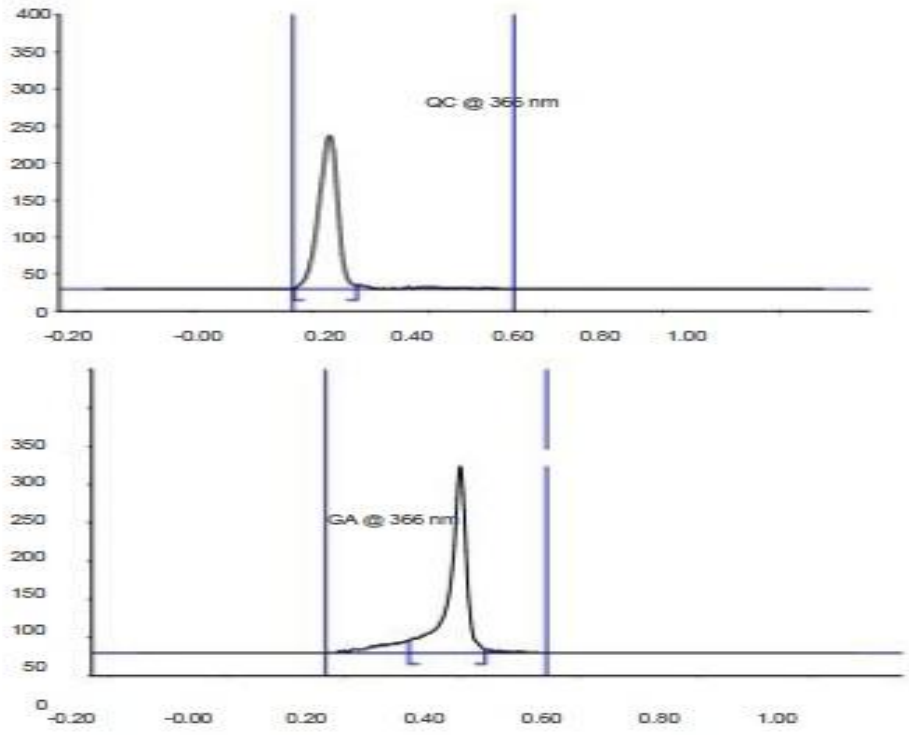


1 2 3 4 5 6 7 8 9 10 11

Photograph of developed TLC plate showing Gallic acid (track 10) and Quercetin (track 11), and methanolic extract of aerial powder of *Anisomeles malabarica* R.BR (tracks 1-9).



A typical HPTLC chromatogram of methanolic extract of aerial powder of *Anisomeles malabarica* R.Br. at λ = 366 nm



A typical HPTLC chromatogram of reference standards Gallic acid and Quercetin at λ = 366 nm

Figure 6

Table 15

Assay of Gallic acid and Quercetin from aerial powder of *Anisomeles malabarica* R.BR

Obs. No	Weight of plant powder (mg)	Peak area of Gallic acid	Amount of Gallic acid found (mg)	Peak area of Quercetin	Amount of Quercetin found (mg)
1	1005	2995	0.360	3558	0.459
2	1001	2990	0.359	3560	0.458
3	1004	2985	0.358	3559	0.459
4	1003	2985	0.358	3560	0.459
5	1005	2995	0.359	3561	0.458
6	1005	2993	0.360	3564	0.459
7	1006	2995	0.359	3563	0.459
Mean	**1004.14**	**2991.1**	**0.360**	**3560.71**	**0.460**
S.D.	**1.676**	**4.561**	**0.0004**	**2.19**	**0.00014**
% R.S.D.	**0.167**	**0.153**	**0.112**	**0.062**	**0.0310**

Calculations of assay of average content of Gallic acid and Quercetin in about 1000.0 mg of aerial powder of *Anisomeles malabarica* R.BR

The linear regression analysis data used for the quantitation of Gallic acid and Quercetin aerial powder of *Anisomeles malabarica* R.Br. is as follows:

$y = mx + c$

Where,

y = mean peak area of Gallic acid and Quercetin

m = slope of the regression line.

c= Intercept on Y-axis.

x = concentration of Gallic acid and Quercetin aerial (µg/band).

From the sample solution, mean peak area of Gallic acid in aerial powder of *Anisomeles malabarica* R.BR, y = 2991.1 **(Table 15)**

From the sample solution, mean peak area of Quercetin in aerial powder of *Anisomeles malabarica* R.Br, y = 3560.7 **(Table 15)**

Mean weight of the sample = 1004.1 mg (**Table 15)**

From the plot of concentration of Gallic acid (X-axis) against peak area of Gallic acid (Y-axis), the linear regression equation obtained for Gallic acid was,

$$y = 5680x - 1093$$ (**Table 7)**

From the plot of concentration of Quercetin (X-axis) against peak area of Quercetin (Y-axis), the linear regression equation obtained for Quercetin was,

$$y = 7518x - 3340$$ (**Table 7)**

Where,

Unknown concentration of Gallic acid in 10.0µL sample solution

$$= (y - c) / m$$
$$= (2991.1 + 1093) / 5680$$
$$= 0.719\mu g.$$

Since, 10.0µL of sample on plate contains 0.719µg of Gallic acid; 5000µL contains 359.5µg of Gallic acid.

Therefore, amount of Gallic acid in 1006.1 mg of leaf aerial powder of *Anisomeles malabarica* R.BR. = 0.360 mg/g.

Unknown concentration of Quercetin in 10.0µL sample solution

$$= (y - c) / m$$
$$= (3560.7 + 3340) / 7518$$
$$= 0.918\mu g.$$

Since, 10.0µL of sample on plate contains 0.918µg of Quercetin; 5000µL contains 458.9µg of Quercetin.

Therefore, amount of Quercetin in 1006.1 mg of aerial powder of *Anisomeles malabarica* R.BR. = 0.459 mg/g.

4.2.1.5 Recovery Experiment

Accuracy of the experiment was determined by recovery experiment at three different levels, using standard addition method. The recovery experiment was carried out to determine if there is any interference of other constituents present in methanolic extract of aerial powder of *Anisomeles malabarica* R.BR with respect to the separation, detection and quantification of Gallic acid and Quercetin.

To the accurately weighed about 1000mg aerial powder of *Anisomeles malabarica* R.BR known amounts of Gallic acid and Quercetin were added in solution form, at three different levels. Each sample solution was then analysed by the developed HPTLC method under optimised chromatographic conditions. (**Table 3)**

The recovery experiment for aerial powder of *Anisomeles malabarica* R.BR was carried out in seven replicates at every level. The amounts of Gallic acid and Quercetin recovered from aerial powder solution for each level were determined. From the amounts of Gallic acid and Quercetin obtained, values of percent recovery were determined for aerial powder of *Anisomeles malabarica* R.BR.

Level Zero

About 1000 mg of aerial powder of *Anisomeles malabarica* R.BR was accurately weighed and sonicated with 10.0 cm^3 of methanol for 15 minutes. The extract was filtered through a Whatman no. 1 qualitative filter paper, pore size 11µm, the contents were then evaporated to dryness and final volume was adjusted to 5.0 cm^3 with methanol in a volumetric flask.

For the determination of Gallic acid and Quercetin from aerial powder solution, the filtrate obtained was used as the test solution. The sample solution was analysed seven times by developed HPTLC method under optimised chromatographic conditions **(Table 3).**

Level 1

About 1000 mg of aerial powder of *Anisomeles malabarica* R.BR accurately weighed and transferred to 10.0 cm^3 standard volumetric flasks.

To the aerial powder of *Anisomeles malabarica* R.BR, 0.360mg Gallic acid and 0.459mg of Quercetin were added in solution form. After addition of known amounts of Gallic acid and Quercetin (as given above) aerial powder of *Anisomeles malabarica* R.BR the contents of flask were diluted up to the mark with methanol.

The contents of flask were extracted with methanol as described for zero level and test solution of aerial powder was prepared as described for zero level. The sample solution was analysed seven times by developed HPTLC method under optimised chromatographic conditions **(Table 3).**

Level 2

About 1000 mg of aerial powder of *Anisomeles malabarica* R.BR was accurately weighed and transferred to 10.0 cm^3 standard volumetric flasks.

To the aerial powder of *Anisomeles malabarica* R.BR, 0.377 mg Gallic acid and 0.476 mg of Quercetin were added in solution form. After addition of known amounts of Gallic acid and Quercetin (as given above) to aerial powder of *Anisomeles malabarica* R.BR, the contents of flask were diluted up to the mark with methanol. The contents of flask were extracted with methanol as described for zero level and test solution of leaf powder was prepared as described for zero level. The sample solution was analysed seven times by developed HPTLC method under optimised chromatographic conditions **(Table 3).**

Level 3

About 1000 mg of aerial powder of *Anisomeles malabarica* R.BR was accurately weighed and transferred to 10.0 cm^3 standard volumetric flasks.

To the aerial powder of *Anisomeles malabarica* R.BR, 0.394 mg Gallic acid and 0.493 mg of Quercetin were added in solution form. After addition of known amounts of Gallic acid and Quercetin (as given above) to aerial powder of *Anisomeles malabarica* R.BR, the contents of flask were diluted upto the mark with methanol. The contents of flask were extracted with methanol as described for zero level and test solution of leaf powder was prepared as described for zero level. The sample solution was analysed seven times by developed HPTLC method under optimised chromatographic conditions **(Table 3).**

The recovery analysis of Gallic acid and Quercetin from aerial powder solutions was carried out on 20 x 10 cm TLC plates. The peak area values of Gallic acid and Quercetin and the amounts of Gallic acid and Quercetin recovered were determined at each level.

The percent recovery was calculated by the following formula,

$$\% \text{ Recovery} = \frac{N(\sum XY) - (\sum X)(\sum Y)}{N(\sum X^2) - (\sum X)^2} \times 100$$

Where,

N = Number of observations.

X = Amount of the standard added (mg).

Y = Amount of the standard found (mg).

The results of recovery analysis of Gallic acid and Quercetin from aerial powder of *Anisomeles malabarica* R.BR are given in Table 16 and 17 respectively.

Table 16

Recovery experiment for Gallic acid after addition of standard Gallic acid to aerial powder of *Anisomeles malabarica* R.BR

Level	Wt. of sample (mg)	Wt. of std. added (mg)	Amount of Gallic acid found (mg)							Mean** (mg)	S.D	% R.S.D
			1	2	3	4	5	6	7			
0	1008	0	0.360	0.359	0.358	0.357	0.360	0.358	0.360	0.359	0.0012	0.34
1	1009	0.360	0.683	0.685	0.684	0.685	0.686	0.686	0.687	0.684	0.0023	0.334
2	1005	0.377	0.723	0.723	0.725	0.723	0.726	0.723	0.725	0.723	0.0017	0.235
3	1006	0.394	0.761	0.765	0.763	0.764	0.762	0.765	0.765	0.763	0.0016	0.212

Table 17

Recovery experiment for Gallic acid after addition of standard Gallic acid to aerial powder of *Anisomeles malabarica* R.BR

Level	X	Y	X^2	XY
0	7 x 0	7 x 0.359	7 $x(0)^2$	7 x 0 x 0.360
1	7x 0.360	7 x 0.684	$7x(0.360)^2$	7 x 0.360 x 0.684
2	7 x0.377	7 x 0.723	$7x(0.377)^2$	7 x 0.377 x 0.723
3	7 x 0.394	7 x 0.763	$7x(0.394)^2$	7 x 0.394 x0.763
∑	**7.917**	**17.70**	**2.99**	**5.74**
$(\sum X)^2$	**62.68**			

No. of observations = 28.

Therefore,

$$\text{Percent recovery} = \frac{(28\times5.74)-(7.92\times17.7)}{(28\times2.99)-(62.68)} \times 100$$

$$= 97.62\%$$

Table 18

Recovery experiment for Quercetin after addition of standard Quercetin to aerial powder of *Anisomeles malabarica* R.BR

Level	Wt. of sample (mg)	Wt. of std. added (mg)	Amount of Quercetin acid found (mg)							Mean** (mg)	S.D	% R.S.D
			1	2	3	4	5	6	7			
0	1007	0	0.457	0.458	0.457	0.456	0.458	0.455	0.457	0.457	0.001	0.234
1	1002	0.459	0.888	0.889	0.890	0.888	0.891	0.893	0.891	0.860	0.08	0.989
2	1007	0.476	0.928	0.926	0.928	0.927	0.926	0.927	0.928	0.927	0.0009	0.097
3	1001	0.493	0.964	0.964	0.965	0.966	0.963	0.968	0.965	0.965	0.002	0.169

* Sample: aerial powder of *Anisomeles malabarica* R.BR.

** Mean amount of Gallic acid and Quercetin found (mg)

Table 4.2.2.19

Recovery experiment for Quercetin after addition of standard Quercetin to aerial powder of *Anisomeles malabarica* R.BR

Level	X	Y	X^2	XY
0	7 x 0	7 x 0.457	$7 x(0)^2$	7 x 0 x 0.457
1	7x 0.459	7 x 0.860	$7x(0.459)^2$	7x 0.459 x 0.860
2	7 x0.476	7 x 0.927	$7x(0.476)^2$	7 x0.476 x 0.927
3	7 x 0.493	7 x 0.965	$7x(0.493)^2$	7 x 0.493 x0.965
∑	**9.99**	**22.46**	**4.76**	**9.18**
$(\sum X)^2$	**99.8**			

No. of observations = 28.

Therefore,

$$\text{Percent recovery} = \frac{(28\times 9.18)-(9.99\times 22.46)}{(28\times 4.76)-(99.8)} \times 100$$

$$= 97.55\%$$

4.2.1.6 RESULTS:

Table 20

Validation parameters for Gallic acid and Quercetin

Parameters	**Gallic acid**	**Quercetin**
Linear working range(µg/band)	0.6-1.3	0.7-1.4
Correlation coefficient(r)	0.999	0.998
Limit of Detection(LOD)(µg/band)	0.6	0.7
Limit of Quantification(LOQ)(µg/band)	0.7	0.8
Repeatability (% R.S.D, n =6) Aerial powder of *Anisomeles malabarica* R.Br	0.05	0.13
Intermediate precision (% R.S.D, n= 18) Aerial powder of *Anisomeles malabarica* R.Br	0.040	0.049
Stability of standard solution	Stable for 24 hours	Stable for 24 hours
System suitability R_f (% R.S.D. , n =6)	1.31	1.84
Peak area (% R.S.D., n=6)	0.013	0.465
Assay (mg/g) Aerial powder of *Anisomeles malabarica* R.Br	0.360	0.459
Percent recovery Aerial powder of *Anisomeles malabarica* R.Br.	97.62%	97.55%

4.2.1.7 Discussion:

In the present research work, a HPTLC method has been developed for the quantitative determination of Gallic acid and Quercetin from aerial powder of *Anisomeles malabarica* R.BR.

Simultaneous quantitation of Gallic acid and Quercetin is not reported in literature. In the present research work, normal mode of separation is used for simultaneous quantitation of Gallic acid and Quercetin from aerial powder of *Anisomeles malabarica* R.BR.

Different combinations of solvents from polar, mid polar and non polar solvents were tried. The solvent strength of Toluene : Ethyl acetate : Formic Acid (4.5 cm^3: 3.0cm^3: 0.5cm^3) v/v/v ratio was adequate to resolve Gallic acid (R_f 0.24) and Quercetin (R_f 0.40) from the other constituents present in the methanolic extract of aerial powder of *Anisomeles malabarica* R.BR.

The peak area of Gallic acid and Quercetin in leaf powder solution increased after addition of standard Gallic acid and Quercetin standard to the leaf powder solution without interference from other peaks. The recovery of Gallic acid and Quercetin was found to be 97.62% and 97.55% respectively indicating good accuracy of the method.

The method used in the present research work was also found to be sensitive to measure the concentrations of Gallic acid and Quercetin as low as 0.6 µg per band and 0.7 µg per band respectively.

4.2.1.8 Conclusion:

An HPTLC method developed for the quantitation of Gallic acid and Quercetin from the aerial powder of *Anisomeles malabarica* R.BR is simple, precise and accurate and can be used for routine quality control analysis of aerial powder of *Anisomeles malabarica* R.BR.

4.2.2 REFRENCES:

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3. Vinitkumar Y. Thakker, Vaishali N. Shah, Uravashi D. Shah, Manish P. Suthar. Simultaneous estimation of Gallic acid, Curcumin and Quercetin by HPTLC method. Journal of Advanced Pharmacy Education & Research 1:70-80 2011.

CHAPTER V
BIOLOGICAL ACTIVITY

INTRODUCTION:

The term “biological” in this background encompass all properties, from for example “abdomen” to “zymase,” which any natural product may possess. And these can be attributed to the whole lively nature, to all living organisms, namely plants, animals, and especially humans.

The “botanical activities” of essential oils (EOs) used by plants for care and interplant communication includes, the prevention of germination of seeds of a potentially rivalry plant by emitting an EO, or the “cry for help” of a plant when it is attacked by pests and the “victim” volatilizes a fragrance which itself attracts enemies of these varmints.

Pheromones, an essential oil, secreted by some animal especially insects act as a messengers that influences the physiology or behaviour of other animals of the same species, as well as the use of EOs as veterinary therapeutics in animal care and feed.

The present chapter deals with the effects of essential oils (EOs) and therapeutic uses of EOs and/or single fragrance compounds in human medicine and care.

The subject matter of this chapter include the pharmacological properties which do not directly aim at the central or autonomic nervous systems and for which the molecular mechanisms are not much more important, for example, anti-oxidative effects and anti-inflammatory activities.

PRESENT RESEARCH WORK:

Arthritis is an auto immune disorder; therefore there are numbers of factors responsible for arthritis. The factors include the damage of connective tissues by formation of free radicals in the body. Inflammation related to tissue which is characterized by swelling, redness and stiffness. There are more than 100 types of arthritis of which the most common are Osteoarthritis, Psoriatic arthritis and Rheumatoid arthritis etc.

Rheumatoid arthritis is a chronic joint disease that damaged the joints of the body. It is also a systemic disease that potentially affects internal organs of the body and leads to disability. The joint damage is caused by inflammation of the joint lining tissues.

Arthritis involves the breakdown of cartilage. Cartilage normally protects a joint, allowing it to move smoothly. Cartilage also absorbs shock when pressure is placed on the joint, such as when you walk. Without the normal amount of cartilage, the

bones rub together, causing pain, swelling (inflammation), and stiffness. The individuals of any age can be affected with Arthritis; the usual age of onset is between 25 and 50 with a peak in the 40s and 50s [1].

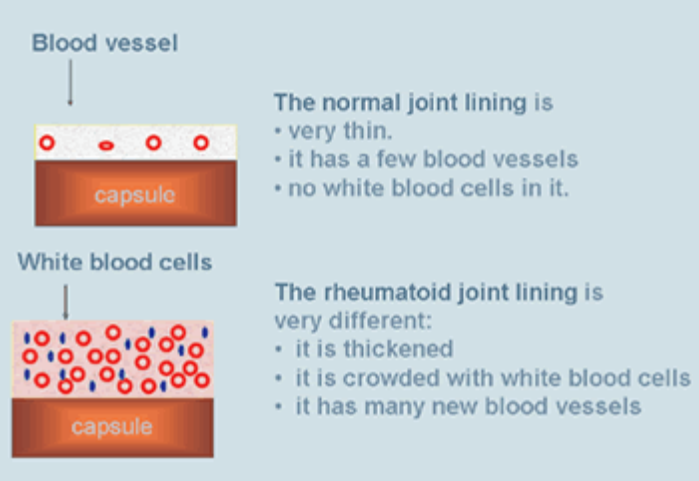

Figure 5: Difference between normal joint lining and rheumatoid joint lining

PRESENT RESEARCH WORK:

For the study of pharmacological actions of the selected plant *Anisomeles malabarica* R.BR; Anti-oxidant and anti-inflammatory activities were studied. Different solvent extracts were used and their potencies were reported along with the essential and fixed oils. Finally a comparison was made with the help of commercial available formulation oil used for local application to reduce arthritis conditions. In-vitro model was selected and how these activities are associated to arthritis was reported.

ABOUT COMMERCIAL OIL SAMPLE:

In the present study two commercial formulation oil samples were used. Their biological activity was determined and compare with the individual plants. Both the formulations are used to reduce the arthritis symptoms.

A) PRASARANI OIL:

1. Prasarani Tail (Oil) is indicated in massage for chronic and acute Rheumatism, Sciatica, Monoplegia, Lumbago, Facial Paralysis, etc.
2. Prasarani Tail (Oil) Ingredients: Prasarini, Yashtimadhu, Piplamul, Chitrak, Sendhava Namak, Gaudugdha, Vacha, Prasarini, Devdaru, Rasna, Gajapipal, Bhilawa, Saunf, Jatamansi.
3. Brand Name: Nagarjun

B) SAHACHARADI OIL:

1. Sahacharadi Tailum is used in relieve sciatic pain and lower back pains.
2. Sahacharadi Tailum Ingredients: Barleria prionities, Aegle marmelos, Oroxylum indicum, Gmelina arboria, Stereospermum suaveolens, Clerodendron phlomoides, Desmodium gangeticum, Uraria picta, Solanum indicum, Solanum xanthocarpum, Tribulus terrestrics, Asparagus racemosa, Vetiveria zizanioides, Helix aspera, Picrorrhiza kurrooa, Santalam album, Elettaria cardamomum, Anisomeles malabarica, Callicarpa macrophylla.
3. Brand Name: Omveda

SECTION I

ANTIOXIDATIVE PROPERTIES

INTRODUCTION:

Numerous physiological and biochemical processes in the human body may produce oxygen-centered free radicals and other reactive oxygen species as by-products. These free radicals are highly unstable, reactive and carry a single electron. They attack other molecules or compounds in the body and become stable thus making possible disorder inside the body. Overproduction of such free radicals can cause oxidative damage to bio-molecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, arthritis, diabetes, aging, and other degenerative diseases in humans [2-3].

To protect the body's own structure from damages, all aerobic living cells use enzymatic and non-enzymatic mechanisms. Enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase are very important in protection mechanisms [4].

Plants (fruits, vegetables, medicinal herbs, etc.) may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity [5-9].

These scavenger molecules produce electrons which dispose free radical and prevent it against damage.

Antioxidants are substances that are able to protect organisms from oxidative stress. Oxidative and anti-oxidative processes should keep the balance. If the balance is in benefit for the oxidative processes, it is called "oxidative stress."

The aim of present study is to find the anti-oxidative properties of selected plant *Anisomeles malabarica* R.BR.

The following antioxidant activities were carried out for different plant part extracts like leaves, flowers. The essential oil extracted from plant was also subjected to evaluate its anti-oxidant potency and a comparison was made with commercial available oils.

5.1.1 DPPH RADICAL SCAVENGING ASSAY:

DPPH is a common acronym for an organic chemical compound 2, 2-diphenyl-1-picrylhydrazyl. It is a dark coloured crystalline powder composed of stable free-radical molecules. DPPH has two major applications, both in laboratory research: one is a monitor of chemical reactions involving radicals, most notably it is a common antioxidant assay [10] and another is a standard of the position and intensity of electron paramagnetic resonance (EPR) signals. DPPH is a well-known radical and a trap ("scavenger") for other radicals. Therefore, rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. Because of a strong absorption band centred at about 520 nm, the DPPH radical has a deep violet colour in solution, and it becomes colourless or pale yellow when neutralized. This property allows visual monitoring of the reaction, and the number of initial radicals can be counted from the change in the optical absorption at 520 nm or in the EPR signal of the DPPH.

5.1.1.1 PREPARATION OF PLANT EXTRACT:

The air dried powdered plant material (10g) were heated in different solvents (150.0mL) like petroleum ether, ethyl acetate, chloroform, methanol and water using Soxhlet apparatus. The extracts were evaporated by rotary evaporator and the residue was collected. For the experimental work petroleum ether, ethyl acetate, chloroform, methanol and water extracts were used.

5.1.1.2 PREPARATION OF STANDARD SOLOUTIONS:

A) 10 mM DPPH solution: 10 mM solution of DPPH was prepared by dissolving 0.1972 g of solid DPPH in 50.0mL of methanol.

B) 1000 PPM Ascorbic Acid (AA) solution: 1000 ppm of ascorbic acid was prepared by dissolving 10 mg of AA in 10.0mL of distilled water.

C) Plant Extract: 1000 ppm of each plant residues of different solvent were prepared by dissolving 10 mg residue in 10.0mL of methanol.

5.1.1.3 PROCEDURE:

1. A 2.0mL DPPH solution was mixed with 500.0µL of plant extract and essential oils.
2. The solutions in the test tubes were shaken well and incubated in the dark for 15 min at room temperature.
3. Thirty minutes later, the absorbance was measured at 515nm using a UV-Visible spectrophotometer (SHIMADZU UV1800).
4. Similarly, commercial available oils and ascorbic acid were treated and their absorbance was measured.
5. The antioxidant capacities of plant extract, essential oils and commercial oils were compared with the standard solution of ascorbic acid.
6. Test control or Blank solution was prepared by mixing 2.0mL of DPPH and 500.0µL of methanol.
7. The percentage scavenging of the radicals due to the antioxidant property of the isolated fractions was calculated using the formula and tabulated.

$$\textbf{\% Scavenging Activity} = \frac{(\boldsymbol{OD\ OF\ CONTROL - OD\ OF\ TEST\ SOLUTION})}{(\boldsymbol{OD\ OF\ CONTROL})} \times \mathbf{100}$$

5.1.1.4 METHOD IN BRIEF:

UV-VISIBLE PARAMETERS	
INSTRUMENT NAME	SHIMADZU UV1800
WAVELENGHT SELECTED λ_{max}	515 nm
BLANK SOLUTION	DPPH + METHANOL

5.1.1.5 OBSERVATIONS:

TABLE 5.1.1.1

DPPH Assay: *Anisomeles malabarica* R.Br

TEST SOLUTION	LEAVES			FLOWERS	
	OPTICAL DENSITY	% SCAVENGING ACTIVITY		OPTICAL DENSITY	% SCAVENGING ACTIVITY
WATER	**0.810**	**42.63**		**0.912**	**35.41**
MEOH	**0.189**	**86.61**		**0.165**	**88.31**
$CHCl_3$	**0.213**	**84.92**		**0.291**	**79.39**
ETAC	**0.275**	**80.52**		**0.257**	**81.79**
PET ETHER	**0.513**	**63.67**		**0.597**	**57.72**
ESSENTIAL OIL	**0.873**	**38.17**		**0.632**	**55.24**
FIXED OIL (AERIAL PART)	**-**	**-**		**0.773**	**45.25**

TABLE 5.1.1.2

DPPH Assay: Standard and Commercial Oil

TEST SOLUTION	DPPH ASSAY	
	OPTICAL DENSITY	% SCAVENGING ACTIVITY
STANDARD ASCORBIC ACID	**0.073**	**94.83**
PRASARANI TAIL	**0.282**	**80.03**
SAHACHARADI TAIL	**0.210**	**85.13**

5.1.1.5 RESULTS AND DISCUSSION:

The present study shows an evaluation of the antioxidant properties present in different extracts of *Anisomeles malabarica* R.Br plant.

In *Anisomeles malabarica* R.Br plants, methanol extracts of leaves and flower shows highest percent scavenging activity. Chloroform and ethyl acetate also shows good anti-oxidant potencies. (Refer table: 5.1.1.1)

The commercial oil sample: Prasarani and Sahacharadi also show maximum activity. The standard ascorbic acid proves to be a great naturally occurring anti-oxidant agent as it shows maximum scavenging activity. (Refer table: 5.1.1.2)

From this it is clear that the plant possesses antioxidant properties and can be used for the treatment of arthritis which is related to free radical production in the body as it scavenges the formation of free radicals.

5.1.2 METAL CHELATING ASSAY:

The metallic elements are the most numerous of the elements and of the metallic elements the d-block elements are the most important because their chemical properties are central to both industry and current research.

One of the most important features of transition metal is the range of oxidation states. This variable oxidation states accounts for the interesting electronic properties of many solid compounds, their ability to participate in catalysis, and their delicate and interesting role in biochemical processes. The ability of transition metals to co-ordinate with certain bio-molecules leads their application to bioinorganic chemist to study other biochemical reactions. The most important examples of transition metal chelation are Vitamin B12, in which Cobalt is the central atom bonded to corrin ring, Haemoglobin in which Iron is chelated with porphyrin ring.

In the present study the chelating capability of transition metal Iron was employed to study the anti-oxidant potency of the selected plants.

Iron occurs in two different stable oxidation states of +3 and +2; both are useful for studying their properties in biochemical process. Iron (III) compounds do not form stable complex with the ligand 1, 10- phenanthroline but in presence of some reducing agent Iron (III) converted to Iron (II) which can bind with the 1, 10-phenanthroline and form stable complex.

5.1.2.1 PREPARATION OF STANDARD SOLOUTIONS:

A) **10.0 mM Ferric ammonium sulphate Fe (III) AS:** 10.0 mM solution of Fe (III) AS was prepared by dissolving 482.19 mg in 100mL double distilled water. From stock 0.5mM was prepared for further study.

B) **1000 PPM Ascorbic Acid (AA) solution:** 1000 ppm of ascorbic acid was prepared by dissolving 10 mg of AA in 10.0mL of distilled water.

C) **Plant Extract:** 1000 ppm of each plant residues of different solvent were prepared by dissolving 10 mg residue in 10.0mL of methanol.

D) **0.25% 1, 10- PHENANTHROLINE:** 0.25 g phenanthroline in 100.0mL of methanol.

5.1.2.2 PROCEDURE:

1. The test solution consisted of 1.0mL of 0.5 mM Fe (III) AS solution and 1.0mL of plant extract and essential oils.
2. To the above solutions 0.5mL of 0.25% 1, 10- phenanthroline and 0.5mL methanol was added.
3. Test tubes were shaken well and incubated for 20 min at room temperature.
4. The absorbance was measured at 515nm using a UV-Visible spectrophotometer (SHIMADZU UV1800).
5. Similarly, commercial available oils and ascorbic acid were treated and their absorbance was measured.
6. The antioxidant capacities of plant extract, essential oils and commercial oils were compared with the standard solution of ascorbic acid.
7. Test control or Blank solution was prepared by mixing 1.5mL of distilled water and 1.5mL of methanol.
8. The percentage chelating activity of the isolated fractions was calculated using the formula and tabulated.

$$\textbf{\% Chelating Activity} = \frac{(\boldsymbol{OD\ OF\ CONTROL - OD\ OF\ TEST\ SOLUTION})}{(\boldsymbol{OD\ OF\ CONTROL})} \times \mathbf{100}$$

5.1.2.3 METHOD IN BRIEF:

UV-VISIBLE PARAMETERS	
INSTRUMENT NAME	SHIMADZU UV1800
WAVELENGHT SELECTED λ_{max}	515 nm
BLANK SOLUTION	DISTILLED WATER + METHANOL

5.1.2.4 OBSERVATIONS:

TABLE 5.1.2.1

Iron chelating Assay: *Anisomeles malabarica* R.Br

TEST SOLUTION	LEAVES			FLOWERS	
	OPTICAL DENSITY	% CHELATING ACTIVITY		OPTICAL DENSITY	% CHELATING ACTIVITY
WATER	**0.880**	**86.14**		**0.406**	**58.70**
MEOH	**0.932**	**87.70**		**1.114**	**91.91**
$CHCl_3$	**0.743**	**80.99**		**0.621**	**74.83**
ETAC	**0.826**	**84.29**		**0.981**	**89.01**
PET ETHER	**0.419**	**59.92**		**0.243**	**39.88**
ESSENTIAL OIL	**0.613**	**74.35**		**0.513**	**67.72**
FIXED OIL	**-**	**-**		**0.418**	**59.30**

TABLE 5.1.2.2

Iron chelating Assay: Standard and Commercial Oil

TEST SOLUTION	Iron chelating Assay	
	OPTICAL DENSITY	% CHELATING ACTIVITY
STANDARD ASCORBIC ACID	**1.403**	**95.84**
SAHACHARADI TAIL	**0.923**	**87.43**
PRASARANI TAIL	**0.896**	**86.64**

5.1.2.3 RESULTS AND DISCUSSION:

The present study shows an evaluation of the antioxidant properties present in different extracts of *Anisomeles malabarica* R.BR plant.

Maximum metal chealting activity was shown by the standard ascorbic acid as it helps in the reduction of Iron (III) to Iron (II). (Refer table: 5.1.2.2)

The commercial oils also show good results of chelation by converting Iron (III) to Iron (II) and their complex formation with the 1, 10- phenanthroline. This indicates the great anti-oxidant potency of commercial oil samples (Refer table: 5.1.2.2).

Anisomeles malabarica R.Br flowers show maximum activity as compared to leaves. Methanolic extracts followed by ethyl acetate and chloroform demonstrate the highest activity. Essential oil also possesses the good anti-oxidant activity which indicates that the oil applied locally to treat arthritis contains chemicals which are responsible for the oxidation reduction process. (Refer table: 5.1.2.1).

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SECTION II

ANTI-INFLAMMATORY PROPERTIES

INRODUCTION:

Processes by which the body reacts to injuries or infections are called inflammations. Inflammations are the complex biological response of vascular tissues to harmful stimuli including pathogens, irritants or damaged cells. It is a protective attempt by the organism to remove the injurious stimuli as well as initiate healing process for the tissue [1]. Inflammation leads to diseases like vasomotor rhinnorhoea, rheumatoid arthritis and atherosclerosis [2].

Inflammation is mainly of two types. They are; Acute inflammation and Chronic inflammation.

Acute inflammation is usually of sudden onset, marked by the classical signs redness, swollen joints processes are predominate.

Chronic inflammation is a prolonged and persistent inflammation marked chiefly by new connective tissue formation; it may be a continuation of an acute form or a prolonged low-grade form. Inflammation is the common clinical conditions and rheumatoid arthritis (RA) is a chronic debilitation auto immune disorder [3].

Mainly Non-Steroidal Anti-Inflammatory Drugs (NSAIDS) are effective for the treatment of pain, inflammation and fever [4]. They are prescribed for orthopaedic conditions such as osteoarthritis, soft-tissue injuries and fractures *etc* [5]. NSAIDs are one of the best classes of drug to prevent and treat postoperative pain [6]. The main issue related to the use of presently available potent synthetic NSAIDs lies in their toxicity and reappearance of symptoms after discontinuation.

The herbal products are rich source for discovery of new drugs because of its chemical diversity and the less toxicity associated with herbal products from medicinal plants play an important role in the development of potent therapeutic agents which can be used to cure many diseases associated with the inflammation.

The present research work aims at the analyses of extracts, essential oils and phytochemicals derived from the selected Indian herbal plants evaluated for the possible anti-inflammatory activity. The research includes the two different In-vitro models for the study of anti-inflammatory potency such as Membrane stabilization Heat Induced Haemolytic test and Inhibition of Protein Denaturation.

5.2.1 HEAT INDUCED HAEMOLYTIC [7-10]

5.2.1.1 PREPARATION OF STANDARD SOLOUTIONS:

A) 1% EDTA (Disodium Ethylene diamine tetracetic acid): 1g EDTA solid dissolved in 100mL distilled water.

B) 1000 PPM Diclofenac Sodium Solution: 1000 ppm of DFS was prepared by dissolving 10 mg of DFS in 10.0mL of methanol.

C) PLANT EXTRACT: 1000 ppm of each plant residues of different solvent were prepared by dissolving 10 mg residue in 10.0mL of methanol.

D) 0.7% Saline solution: 0.7 g sodium chloride in 100.0mL of distilled water.

E) 10% V/V RBCs Suspensions: Fresh whole human blood (20 ml) was collected and 2.0mL of anti-coagulating agent 1% EDTA solution was added. The bloods were transferred to the centrifuge tubes and were centrifuged at 3000 rpm for 15 minutes and were washed three to four times with equal volume of 0.7% normal saline solution and again centrifuged. Finally the volume of blood was measured and re-constituted as 10% v/v suspension with normal saline.

5.2.1.2 PROCEDURE:

1. The reaction mixture consisted of 2.0mL of test sample solutions and 2.0mL of 10% RBCs suspension.
2. For control 2.0mL of 10% RBCs suspension and 2.0mL of 0.7 % Saline were added.
3. 2.0mL of Diclofenac sodium and 2.0mL of 10% RBCs suspension was taken as standard.
4. All the solution tubes containing reaction mixture were incubated in water bath at 56°C for 30 min.
5. The reaction mixture was cooled and centrifuged at 2500 rpm for 5 minutes.
6. The absorbance of the supernatants was measured at 560nm using a UV-Visible spectrophotometer (SHIMADZU UV1800).
7. The experiment was performed in triplicates for all the test samples. Percent membrane stabilization activity was calculated and tabulated.

$$\% \text{ Membrane Stabilization} = \frac{(OD\ OF\ CONTROL - OD\ OF\ TEST\ SOLUTION)}{(OD\ OF\ CONTROL)} \times 100$$

5.2.1.3 METHOD IN BRIEF:

UV-VISIBLE PARAMETERS	
INSTRUMENT NAME	SHIMADZU UV1800
WAVELENGHT SELECTED λ_{max}	560 nm
BLANK SOLUTION	SALINE SOLUTION

5.2.1.4 OBSERVATIONS:

TABLE 5.2.2.1

Heat Induced Haemolytic Assay: *Anisomeles malabarica* R.Br

TEST SOLUTION	**LEAVES**			**FLOWERS**	
	OPTICAL DENSITY	**% MEMBRANE STABLIZATION**		**OPTICAL DENSITY**	**% MEMBRANE STABLIZATION**
WATER	**0.512**	**40.33**		**0.416**	**51.52**
MEOH	**0.174**	**79.72**		**0.044**	**94.87**
$CHCl_3$	**0.253**	**70.51**		**0.210**	**75.52**
ETAC	**0.306**	**64.33**		**0.089**	**89.63**
PET ETHER	**0.313**	**63.52**		**0.263**	**69.35**
ESSENTIAL OIL	**0.372**	**56.64**		**0.298**	**65.27**
FIXED OIL	-	-		**0.426**	**50.35**

TABLE 5.2.2.2

Heat Induced Haemolytic Assay: Standard and Commercial Oil

	Heat Induced Haemolytic Assay	
TEST SOLUTION	**OPTICAL DENSITY**	**% MEMBRANE STABLIZATION**
STANDARD DICLOFENAC SODIUM	**0.188**	**78.09**
SAHACHARADI TAIL	**0.147**	**82.87**
PRASARANI TAIL	**0.120**	**86.01**

5.2.1.5 RESULTS AND DISCUSSION:

The heat inducing haemolytic assay shows that the how the effect of heat can lead to damage the cells which is indicated by the sign of inflammation either swelling or redness. The inflammation symptom related to the arthritic disease.

In the present study inflammation action of different extracts of *Anisomeles malabarica* R.BR plant were studies by treating the plants extracts with the human red blood cells and how the heat can affect the inflammation was studied.

The methanolic extract of *Anisomeles malabarica* R.BR flowers shows maximum heat induced as compared to the commercial oil samples. The standard diclofenac sodium shows moderate heat inducing activity. (Refer table: 5.2.2.1 and 5.2.2.2)

5.2.2INHIBITION OF PROTEIN DENATURATION [11-13]

5.2.2.1 PREPARATION OF STANDARD SOLUTIONS:

A) 0.5% W/V Bovine Serum Albumin (BSA):0.5% BSA solution**:** 0.5g BSA solid dissolved in Tris buffer saline and pH was adjusted to 6.8 using hydrochloric acid.

B) 1000 PPM Diclofenac Sodium Solution: 1000 ppm of DFS was prepared by dissolving 10 mg of DFS in 10.0mL of methanol.

C) Plant Extract: 1000 ppm of each plant residues of different solvent were prepared by dissolving 10 mg residue in 10.0mL of methanol.

5.2.2.2 PROCEDURE:

1. Test solutions consisted of 0.5mL each extract and 5.0mL of 0.5% W/V BSA.
2. The control consists of 5.0mL 0.5% W/V BSA solution with 0.5mL methanol.
3. The standard consist 0.5mL of Diclofenac Sodium in methanol with 5.0mL 0.5% W/V BSA solution.
4. The sample extracts, standard and control were incubated at 37°C for 20 min and then heated to 51°C for 10 minutes.
5. The reaction mixture was cool and the turbidity was measured spectrophotometrically at 660 nm.
6. The experiment was performed in triplicates for all the test samples. The % inhibition of precipitation (denaturation of the protein) was determined and tabulated.

$$\textbf{\% Inhibition} = \frac{(\boldsymbol{OD\ OF\ CONTROL - OD\ OF\ TEST\ SOLUTION})}{(\boldsymbol{OD\ OF\ CONTROL})} \times \mathbf{100}$$

5.2.2.3 METHOD IN BRIEF:

UV-VISIBLE PARAMETERS	
INSTRUMENT NAME	SHIMADZU UV1800
WAVELENGHT SELECTED λ_{max}	560 nm
BLANK SOLUTION	SALINE SOLUTION

5.2.2.4 OBSERVATIONS:

TABLE 5.2.2.1:

Protein Inhibition Assay: *Anisomeles malabarica* R.Br

TEST SOLUTION	LEAVES			FLOWERS	
	OPTICAL DENSITY	% INHIBITION		OPTICAL DENSITY	% INHIBITION
WATER	**0.413**	**59.44**		**0.503**	**67.04**
MEOH	**0.987**	**89.19**		**0.909**	**87.06**
$CHCl_3$	**0.809**	**83.71**		**0.874**	**85.96**
ETAC	**0.819**	**84.08**		**0.850**	**85.17**
PET ETHER	**0.639**	**75.90**		**0.893**	**86.58**
ESSENTIAL OIL	**0.537**	**69.52**		**0.512**	**67.72**
FIXED OIL	-	-		**0.501**	**66.88**

TABLE 5.2.2.2

Protein Inhibition Assay: Standard and Commercial Oil

TEST SOLUTION	Protein Inhibition Assay	
	OPTICAL DENSITY	% INHIBITION
STANDARD DICLOFENAC SODIUM	**0.982**	**89.06**
SAHACHARADI TAIL	**0.876**	**86.04**
PRASARANI TAIL	**0.908**	**87.03**

5.2.2.5 RESULTS AND DISCUSSION:

The denaturing of proteins by heat leads to the symptoms of inflammation. The structural change in protein may be secondary or tertiary. The change in structure may be by many factors like change in pH, heat etc.

In the present study, the structure of proteins was studied by treating bovine serum albumin and adjusting the pH and applying heat to the test solutions.

It was observed that the both the plant *Anisomeles malabarica* R.BR inhibits the deformation of protein which proves that the plants possess some active phytoconstituents which act as an inflammatory agent.

A slightly higher inhibition of protein denaturation and proteinase inhibition was observed in the present study. The production of auto antigen in certain arthritic diseases may be due to the denaturation of protein. This effect may be due to the presence of steroids, alkaloids and flavonoids. The mechanism of denaturation involves alteration of electrostatic hydrogen, hydrophobic and disulphide bonding [14]. Proteinases have also been implicated in arthritic reactions. It was previously reported that leucocytes proteinases play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors [15].

It was found that the methanolic extracts of both the plants have good inhibiting properties. (Refer table: 5.2.2.1, 5.2.2.2)

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CHAPTER VI

GENERAL CONCLUSION

CONCLUSIONS:

In physicochemical analysis of *Anisomeles malabarica* R.BR, the highest extractive value was obtained from water and Methanol. High alcohol soluble and water soluble extractive values reveal the presence of polar substance like phenols; tannins and glycosides.

In qualitative phytochemical analysis, phenolics, flavonoids, and alkaloids class of compounds were present in high amount as compared to other phytoconstituents analyzed. Hence, the determination of physico and phytochemical profile of *Anisomeles malabarica* R.BR may be useful to supplement information in respect to its identification, authentication and standardization of herbal drugs.

The solubility of the extract was maximum in polar solvents like, methanol, chloroform and ethyl acetate.

During extraction of essential oil two techniques Steam distillation and Hydro-distillation were employed. The investigation of the effect of percent yield obtained from these techniques shows that Hydro-distillation method is superior over steam distillation.

For fixed oil extraction hot continuous extraction method i.e. Soxhlet Extraction method were used. The percentage amount yield of fixed oils present in the leaves shows high percent content of fixed oil in the selected plant *Anisomeles malabarica* R.BR.

Physicochemical analysis of essential and fixed oil extracted from *Anisomeles malabarica* R.BR shows great variation. The essential oils of *Anisomeles malabarica* R.BR was found to be pale yellow in colour and fixed oil was colourless. Both the volatile had pleasant odour while fixed oil had unpleasant odour.

Fixed oils have high specific gravity values as compared to essential oils of both plants. The optical rotation was found to be high in fixed oils of both the plants.

Saponification values determined and found to be very high in *Anisomeles malabarica* R.BR. All the oil samples are found in the range reported for plants (lower than the 188-196) but not useful in soup industry as these values are much lesser than the required value (±300).

The iodine valve observed for the oil samples in the present study were between 100-160g/100g oil. These values were found in the permissible range for semi-drying of oil (100-300). Recorded data for iodine number showed that all the oil samples have high unsaturated fatty acids contents.

In Chromatographic analysis, Gas Chromatography Mass Spectrometry methods were developed for the extracted essential and fixed oils from the selected plant *Anisomeles malabarica* R.BR. The developed GCMS method can be used for the identification of essential and fixed oil constituents from these plants as a quality parameter.

The present research work has been concerned with determining and comparing the chemical compositions of essential oil distilled from the *Anisomeles malabarica* R.BR plants. The chemical analysis by GCMS have allowed us to identified total 38 essential oil components from the *Anisomeles malabarica* R.BR leaves and flowers. This shows that the *Anisomeles malabarica* R.BR plants have more essential oil components.

The fixed oils detected by GCMS were high in *Anisomeles malabarica* R.BR flowers.

The biological activities associated with the constituents identified by GCMS were compared by Dr. Duke's database and Pubmed data available from the previously isolated components of different plants.

Simultaneous HPTLC method were developed and validated for Gallic acid and Quercetin in *Anisomeles malabarica* R.BR. The developed HPTLC methods are simple, accurate and precise and can be used for regular quality control analysis of aerial powders of these plants.

Biological activities of different extracts of both the plant were evaluated using In-vitro model.

DPPH assay reaction depends on the ability of the samples to scavenge free radicals which is visually noticeable as the colour change from purple to yellow due to hydrogen donating ability. The more rapid the absorbance decrease, the more potent the primary antioxidant activity. Methanolic extracts of both the plants shows highest scavenging activities compare to others. It is known that only flavonoids with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and show radical scavenging activity. This can be confirmed by the phytochemical analysis data where methanol extracts shows maximum positive test.

In reducing power assay, ferric is reduced to ferrous and form complex with phenanthroline. This indicates presence of certain class of compounds which shows as an oxidising and reducing agents. Mostly flavonoids and steroid compounds show redox properties.

All the extracts were effectively inhibiting the heat induced hemolysis. These results provide evidence for membrane stabilization as an additional mechanism of their anti inflammatory effect. This effect may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophils lysosomal constituents include bactericidal enzymes and proteinases, which upon extracellular release cause further tissue inflammation and damage.

A slightly higher inhibition of protein denaturation and proteinase inhibition was observed in the present study. The production of auto antigen in certain arthritic diseases may be due to the denaturation of protein. This effect may be due to the presence of steroids, alkaloids and flavonoids. The mechanism of denaturation involves alteration of electrostatic hydrogen, hydrophobic and disulphide bonding. Proteinases have also been implicated in arthritic reactions. It was previously reported that leucocytes proteinases play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors.

These studies have shown that the methanol extract of *Anisomeles malabarica* R.BR contain some active ingredients with the potential of being good anti-inflammatory and antioxidant agents. NSAIDs like diclofenac, used as standard drug in anti-inflammatory study, is having good anti-inflammatory and analgesic property, but is also having side effects on liver. Therefore *Anisomeles malabarica* R.BR may become the alternative to the NSAIDs. For that, further study for detailed investigation of the mechanism of action is needed.