EFFECTS OF ACOUSTIC WAVES ON THE GROWTH, DEVELOPMENT AND PHYTOCHEMICAL CONTENTS OF *IN VITRO* RAISED *SWERTIA CHIRAYITA*

Dissertation submitted in partial fulfillment of the requirement for the degree of

MASTERS OF SCIENCE
IN
BIOTECHNOLOGY

BY

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2023

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DECLARATION

I hereby declare that the work presented in this report entitled **"Effects of Acoustic waves on the Growth, Development and Phytochemicals contents of *in vitro* raised *Swertia chirayita*"** in partial fulfillment of the requirements for the award of the degree of "**Masters** in **Biotechnology"** submitted in the Department of Biotechnology & Bioinformatics**, "**Jaypee University of Information Technology Waknaghat", is an authentic record of my own work carried out over a period from January 2023 to May 2023 under the supervision of **Dr. Hemant Sood** (Supervisor) and **Dr. Poonam Sharma** (Co-supervisor). The matter embodied in the report has not been submitted for the award of any other degree or diploma.

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SUPERVISOR'S CERTIFICATE

This is to certify that the project work titled **"Effects of Acoustic waves on the Growth, Development and Phytochemicals contents of *in vitro* raised *Swertia chirayita*"** by **Swalpanaa** during her end semester in fulfillment for the award of degree of Master's in Biotechnology of Jaypee University of Information Technology, Solan, has been carried out under my supervision. This work has not been submitted partially to any other University or Institute for the award of any degree or appreciation.

Signature of Supervisor- _____ _____

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ACKNOWLEDGEMENT

I take this opportunity to express my first and foremost gratitude to the "DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS" for showing confidence in me and entrusting my project **"Effects of Acoustic waves on the Growth, Development and Phytochemicals contents of *in vitro* raised *Swertia chirayita*"**

I feel honored in expressing my sense of gratitude and deepest appreciation to Guide **Dr. Hemant Sood (Associate Professor)** and Co-Guide **Dr. Poonam Sharma (Associate Professor)** for believing me, providing guidance and motivation throughout the time. I thank them of being continuous source of inspiration for me and for giving insightful advices and suggestions. The help and support I got from their side helped me to successfully complete my project on time.

The in-house facilities provided by the department throughout the Masters programme are also equally acknowledgeable. I would like to convey my thanks to **Dr. Sudhir Syal, HOD,** Dept. of Biotechnology and Bioinformatics, Jaypee University of Information Technology**.**

I would like to extend my thanks to **Mrs. Mamta Mishra (Lab Technician), Miss. Rolika Gupta (PhD Scholar)** and other staff of the department for their invaluable help and support. And last but not least, I would like to thank all my friends especially Shalini Thakur, Alka Sharma, Saurabh Pandey who helped me in different aspects of this project.

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ABBREVIATIONS

PAFT	Plant acoustic frequency technology
PGR	Plant growth regulators
MS (Media)	Murashige and Skoog
LAF	Laminar air flow
TPC	Total phenolic content
TFC	Total flavonoids content
DPPH	Diphenyl-1-picrylhydrazyl
RSA	Radical scavenging activity
Hz	Hertz
kHz	Kilohertz

ABSTRACT

Swertia chirayita, a medicinal herb that is present at the temperate Himalyas and is most popular herb because of its large no. of pharmacological activities. This plants contains various chemical constituents i.e. mangiferin, amarogentin, swertiamarin, gentianine, swerchirin etc. These chemical constituents contains many medicinal uses such as it is antidiabetic, hepatoprotective, anticancer, antiviral, antiinflamatory, antimicrobial, antimalarial, etc. According to reports, sound affects the growth, development and phytochemical contents of plants. So, in the present study, two aspects are studied i.e. effects of acoustic waves were checked on the plant growth & its chemical constituents and acoustical parameters study of *S.chirayita* extracts. Sound exposure was provided to some plants at different frequencies, i.e. 500Hz, 1kHz and 1.5kHz. The plant showed better and faster growth in shoots of sound exposed plants. Sound exposed plants also gave better results in total phenolic contents and total flavonoids contents. Total phenolic content (TPC) in control plant was 1.411mg GA g^{-1} while in test plant having frequency 500Hz, 1kHz and 1.5 kHz it was 1.315mg GA g^{-1} , 1.486 mg GA g^{-1} and 1.453mg GA g^{-1} respectively. It was more in 1kHz and 1.5kHz plants but less in 500Hz plants than the control plants. Total flavonoids contents (TFC) was also increased and was in more quantity in test plants than the control plants.TFC was 0.106mg QE g^{-1} , 0.14mg QE g^{-1} , 0.19mg QE g^{-1} and 0.164 mg QE g^{-1} in control, 500Hz, 1kHz and 1.5kHz respectively. Antioxidant activity by DPPH assay was also higher in 1kHz i.e. 64.68% RSA while less was in control plant having 47.39% RSA. At 500 Hz and 1.5kHz RSA were 54.40% and 57.75% respectively. In this study, acoustical parameters studied in *S. chirayita* liquid extract says that the rise in temperature changes the ultrasonic velocity, viscosity, adiabatic compressibility, density, relaxation time andacoustic impedance. These changes can be helpful for the physicochemical studies of liquid extracts.

Keywords: *S. chirayita*, hepatoprotective, gastroprotective, acoustic waves, ultrasonic interferometer, viscometer, adiabatic compressibility, acoustic impendance, plant acoustic frequency technology, PAFT

CHAPTER 1

INTRODUCTION

1.1 Basic Introduction

Swertia is a large genus of annual and perennial herbs with over 135 species, belonging to the Gentianaceae family. Several herbal treatments are done by these *Swertia* species. There are 40 species of *Swertia* found in India, with *Swertia chirayita* being the most significant due to its medicinal qualities. A medicinal plant known as *S. chirayita*, sometimes known as "Chiretta," thrives at high elevations on the slopes of moist, gloomy locations in the sub-temperate Himalayan highlands between 1200 and 3000 m heights from Kashmir hills to Bhutan [2]. Many different ailments can be treated with them. In Indian medicine, these plants are used to treat bronchial asthma, diarrhea, liver disorders, anaemia and recurrent fevers. *S. chirayita* is used in Ayurveda as a laxative, antihelmintic, antipyretic, and to cure leucorrhea and asthma. It functions as an anti-inflammatory, astringent and tonic in Unani herbal remedies. Several components of *S. chirayita* are thought to be effective medicines, but the roots are the best-known component [35].

Fig 1. ***Swertia chirayita***

1.2. Medicinal uses of *S. chirayita*

This plant has various pharmacological activities such antimalarial, antidiabetic, anticancer, antiviral, antitumor, anti-inflammatory, antimalarial, gastroprotective, antihypoglycemic, hepatoprotective, and many more other activities that makes this plant most important in field of research [1].

1.3. Acoustic waves on plants and its extracts

Reports says that acoustic waves have positive effects on plants growth and its development. An acoustic wave, also known as a sound wave, is often an oscillation of mechanical pressure that flows through a solid, gas, plasma or liquid via a periodic wave pattern and transfers energy in medium from one point to another. These are a type of longitudinal wave that travels through a medium mostly air using compression and decompression also known as rarefactions. These waves vibrate in the wave's direction of travel because they move at the speed of sounds therefore it is called acoustic. It also goes by the name "acoustic signal" since it conveys sound by causing the ear's auditory organs to vibrate. These waves must originate from vibrating material in the medium and take the transmission form of compression and rarefaction. It is always impossible for sound to spread in the absence of any physical medium [10]. Various studies have suggested that acoustic waves acts as a stress on plant which helps a plant to grow and develop very fast. Plant absorbs some frequencies that help to produce more mRNA and changes the rate of transcription. Acoustic waves not only affects the plant growth but also increases the phytochemical constituents that plant consists [29]. The molecular interactions among these phytochemical constituents in the plant extract can be studied with the help of acoustical parameters. Ultrasonic velocity, viscosity and density are helpful to study the molecular interaction and acoustical parametes between the solute and solvent at different temperatures.

Therefore, in this study two parameters were checked. First one was to know how sound affects the growth, phytochemical contents and development of a plant; and another one was acoustical study of those plant extracts in order to study the molecular interaction. Effects of sound waves has not been checked on the medicinal plants yet. Thus in the present study, effects of sounds waves on a medicinal plant i.e. *Swertia chirayita* was checked.

1.4. Objectives

1. To carry out *in vitro* propagation of *S. chirayita* by using shoot apices.
2. To check the effects of acoustic waves on growth, development and production of phytochemicals of *in vitro* raised *S. chirayita.*
3. To study acoustical parameters of *in vitro* raised *S. chirayita* plant extracts.

CHAPTER 2

REVIEW OF LITERATURE

2.1. *Swertia chirayita*

Swertia chirayita is a medicinal herb and according to The National Therapeutic Plant Board, Govt of India, this plant has been ranked as one of the top 32 therapeutic herbs [3]. The primary characteristics of this medicinal herb is its bitter flavour, which is brought on by the existance of various chemical components including amarogentin (responsible for bitterness), swertiamarin, swerchirin, and other bioactive ingredients that are directly linked to the improvement of human health [1].

S. chirayita is 0.6 to 1.5 m tall and has an upright, two to three feet long stem, with upper portion that is rectangular and a central portion extending downwards long with the stem at each angle. Its stem has a big, continuous golden pith and is purplish, brown, orange in hue [2]. They are cylindric, occasionally quadrangular, orange-brown with purplish colouring, and contain a thick, continuous layer of yellow pith. Opposite-paired, , septate, cordate at the base, without stalks, lanceolate, 4 cm long , sessile and five to seven nerved are the characteristics of the leaves. The root is half inch thick , approximately 7-8 cm long, curving, somewhat geniculate and yellowish. The soil types that this plant prefers include clay (heavy), sandy (light) and loamy (medium). In addition, the this plant does well in basic or alkaline soils as well as acidic, neutral, and basic soil [3].

2.2. Chemical Constituents of *S. chirayita*

Amarogentin, Swertiamarin, Swerchirin, Mangiferin, Ursolic acid, Oleanolic acid, Gentianine, Amaroswerin, and Sweroside are the primary chemical components found in *Swertia*. The presence of flavonoids, xanthones, glycosides, terpenoids, steroids, and ascorbic acid is also confirmed in this plant by phytochemical analyses [5]. The biological activity of the *Swertia* genus includes hepatoprotective, antimicrobial, anti-inflammatory, anti-carcinogenic, anti-leprosy, antimalarial, antioxidant, anti-cholinergic, CNS depressing, and mutagenic properties. *Swertia'*s pharmacological attributes have generated a lot of curiosity in researches for medicinal properties[4].

Table 1. Chemical constituents of *Swertia chirayita* and their medical uses

S.NO.	CHEMICAL CONSTITUENTS	MEDICINAL PROPERTIES
1.	Amarogentin	Anticancer Gastroprotective Anti-diabetics[1]
2.	Amaroswerin	Gastroprotective [4]
3.	Gentianine	Antipsychotic Antimalarial [4]
4.	Mengiferin	Antiviral Anti-HIV Antioxidants Chemo-preventive Anti-inflammatory Hypoglycemic Anti-diabetic [4]
5.	Oleanolic acid	Antimicrobial Antitumor Antioxidant Anti-inflammatory[3]
6.	Swerchirin	Hypoglycemic Hepatoprotective Blood glucose lowering activity Chemopreventive[3]
7.	Sweroside	Antibacterial Hepatoprotective Hyperpigmentation[3]
8.	Swertiamarin	CNS depressant Anticancer Anti-hepatitis Antibacterial Cardioprotective Antidiabetic Anti-arthritic Anticholnergic [3]
9.	Ursolic acid	Antimicrobial[4]

2.3. Acoustic waves on plants

Sound is acoustic energy that travels through gases, liquids, and solids as an oscillating concussive pressure wave. A medium, such as liquid water, gaseous air, or a solid substance, is what allows sound, which is a vibration, to travel through space. A sound source emits vibrations into the medium around it, which in turn produces sound waves. As the medium continues to vibrate in response to the sound source, the sound wave is created as the vibrations travel as quickly as sound away from the source. Transverse and longitudinal waves are both used in the transmission of sound through solid objects and through water, air, and other media. While particles in transverse waves oscillate in a direction that is at a right angle to that of propagation, those in longitudinal waves oscillate in a direction that is parallel to that direction. The three characteristics of sound waves—frequency (Hz), strength (dB), and timbre—distinguish one sound from another at the same frequency [7]. The fact that plants respond to sound (a physical force) by producing more mRNA suggests that sound alters plant transcription. In fact, following 125 and 250 Hz sound therapy, rise particularly stimulated two genes i.e. rubisco small sub-unit (rbcS) and fructose 1,6-bisphosphate aldolase (ald), which are essential for photosynthesis. Constant sound exposure is believed to improve plant development by encouraging CO_2 fixation [29]. Sound waves vibrate plant leaves, speeding up protoplasmic activity in cells. Certain sound frequencies may be absorbed and reflected by plants, affecting the cell cycle. Although the biological impacts of sound have been researched in the past, it is still unclear how sound interferes with plant growth. According to a study, sound stimulation can activate numerous stress-related genes at the transcriptional level. The activation of sound waves may also enhance the amount of amylase activity, soluble proteins and soluble sugars, in the callus, as well as the activity of the plant plasma-membrane H^+ ATPase. Sound vibrations can modify the activity of certain proteins, increase levels of polyamines and soluble carbohydrates, reorganise microfilaments, and regulate the transcription of specific genes [8]. Numerous plant components, including hormones, alkaloids, flavonoids, phenols, terpenes, quinones, etc., can undergo modifications as a result of sound waves. It causes alterations in the activity of enzymes, antioxidants, gene expression, and protein synthesis as well as different stress-related hormones. Some of these might be concerned with human nutrition, and safe processing creates a number of opportunities for enhancing the beneficial properties of plant-based foods [9].

2.3.1. PAFT

Plant Acoustic Frequency Technology, also named as PAFT is a method that uses acoustic waves to encourage plant growth. This acoustics is used to induce the opening of stomata in plants, increasing the entry of nutrients and other material that is needed by plants to flow via the stomata. Thus it is able to increase the results of substances required in the process of plant growth [28]. This technology includes a device that produces various frequencies that can be provided to the plants through which it can be checked whether the plants are affected by sound frequency or not. A frequency modulator (function generator) (fig.2) connected to a speaker to produce various frequencies can be useful to expose plants under different frequencies in a closed soundproof chamber. A signal generator called a "function generator" creates several waveforms as an output. It can create waveforms like square waves, triangular waves , sine waves, and more. The range of frequencies in a function generator is between a few Hz and several hundred kHz [30].

Fig 2. Function Generator

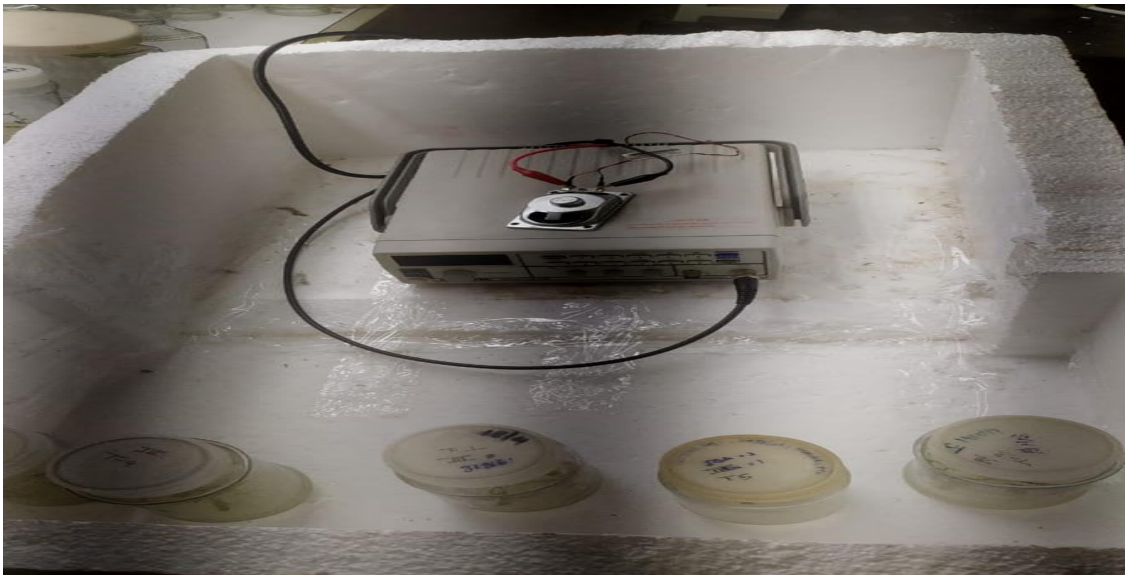


Fig 3. PAFT Technology

2.4. Acoustic waves on plant extract

Plants have a very good effect of sound on their growth and development. Just like plants, acoustic waves can also affects plant extracts. Other than medicinal uses of plants they can be studied for the knowledge of chemical constituents that can be helpful for the drug development with its selectivity precisely. The liquid extract of plants can be made to study the physiological properties of plants thus molecular interaction in liquid extract can be studied. These interactions can be studies by exposing extract in instruments i.e. ultrasonic interferometer, viscometer, density meter etc and through this various acoustical parameters can be studied. Measurements of acoustical parameters are a crucial technique for researching liquid states. It is possible to learn important details about the physico-chemical characteristics of liquid mixtures as well as the nature of molecular interactions in them by measuring the acoustical parameters i.e. velocity, viscosity, density, adiabatic compressibility, free length in the mixture. Understanding different intra- and intermolecular interactions in solution is made possible by ultrasonic parameters [25]. After treating the liquid with acoustic waves there are various acoustical parameters that are calculated to check the molecular interactions in that liquid.

2.4.1. Ultrasonic Interferometer

An Ultrasonic Interferometer is a straightforward instrument that can accurately measure the ultrasonic velocity in liquid (fig 4). In order to fully understand intramolecular and intermolecular interactions, physiochemical behaviour, and structural behaviour, as well as to validate various liquid state theories that make an attempt to determine the characteristics of liquid mixtures, it is crucial to understand the ultrasonic, thermophysical, and thermodynamic properties of liquid mixtures. The ultrasonic waves in an ultrasonic interferometer are created using piezoelectric techniques . The wavelength of the sound in a liquid sample is measured at a fixed frequency variable path interferometer, and from this one can determine the velocity of the sound through that medium [11]. It is possible to determine the sound's velocity through a liquid experimental medium by measuring the wavelength of the sound at a fixed frequency variable path interferometer. A quartz crystal present in the bottom of the cell generates ultrasonic waves with predetermined frequencies. The quartz plate is parallel to a moving metallic plate that reflects the waves. Standing waves are created in the liquid medium when the waves interact with their reflections and the distance between the plates is an integer multiple of the half-wavelength of sound. Acoustic resonance happens in these conditions. The

anode current of the piezoelectric generator reaches its maximum when the amplitude of the resonant waves reaches its maximum. The major components of the ultrasonic interferometer are - Measurement cell and High frequency generator[11].

2.4.2. Viscometer

Viscometer is a device that is used to measure the flowing behaviour of a liquid. The viscosity of thin and thick liquids is often measured using a viscometer (fig 5). Rotational viscometer is widely used among various types. These viscometers work on the basis of "rotational viscometry," which involves immersing a carefully chosen spindle in the sample in order to measure the torque needed to spin the spindle at a predetermined speed while immersed in the product sample. This offers a measurement of the product viscosity, measured in centipoises units (cP), since the torque needed will be proportional to the amount of viscous drag on the spindle. A cup filled with the test liquid is put inside of a bob with a circular cross section. A torque sensor measures the liquid's pull on the bob as it is turned. Since the instrument typically has the ability to change the shear rate, it is possible to produce the shear stress against shear rate plot to describe the liquid and determine the flow parameters [36].

Fig. 4. Ultrasonic Interferometer

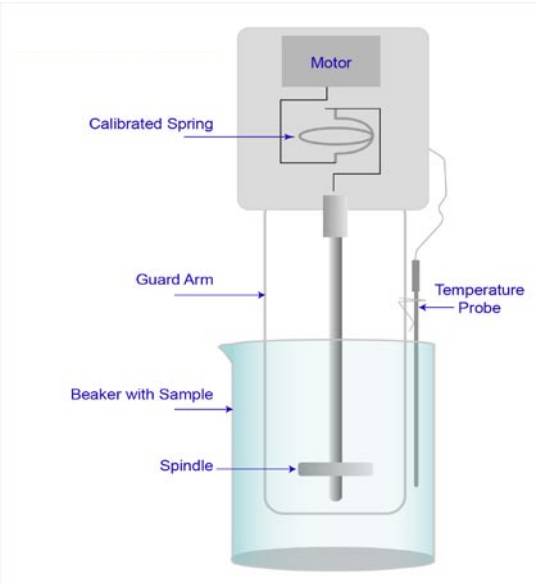


Fig. 5. Viscometer

2.5. Effects of acoustic waves on plant growth and its chemical constituents

Sound can increase the growth of plants positively. In Red Young Radish (*Raphanus sativus*) , Broccoli *(Brassica oleracea*) and Alfalfa (*Medicago sativa*), flavonoid production was examined in response to different sound wave exposure(250 Hz - 1.5 kHz). In accordance with the growth stages, species, sound wave frequency, and exposure time, the results demonstrated that sound wave treatments significantly impacted the total flavonoid contents. In compared to the untreated control, the total flavonoid content of red young radish sprouts (1 kHz), broccoli sprouts (800 Hz) and alfalfa (250 Hz were all enhanced by 200%, 35%, and 85%, respectively by sound wave treatments. According to a molecular analysis, the flavonoid content positively correlates with sound waves' induction of the expression of genes involved in the process that produces flavonoids [23].

The results of the PAFT test on mustard pakcoy were able to increase its average height by 10,4%, stomatal opening by 28,4%, potassium (K) absorption by 34,42%, absorption of nitrogen (N) content by 25,36%, leaf area by 30,9% and total chlorophyll content higher by 27,7% on exposed plants [28].

Noise is unrythemic and unharmonised superposition of various audio frequency that showed negative effects to plant . This was checked by Marigold plant exposed to light music (20Hz-20kHz) by which the plant growth was fast than untreated. Marigold exposed to noise i.e. above 20kHz and in -1st week- Similar growth in treated and untreated. In 2nd week- Bud no. started decreasing in treated and In 3rd week- Plants started dying in treated [26].

2.6. Effects of acoustic waves on plant extracts

Brayophallum leaves were extracted in water using a Soxhelt extractor, and then its viscosity, density and ultrasonic velocity were assessed at various temperatures. Understanding the nature of molecular interaction in terms of physical parameters can be done with the help of information derived from ultrasonic propagation parameters such as free length, adiabatic compressibility, ultrasonic velocity, acoustic impedance, and their variation with temperature. The results demonstrated that as temperature rose, the ultrasonic velocity of a leaf extract from Brayophallum decreased, whereas free length, adiabatic compressibility, acoustic impedance, and relaxation time increased, indicating weak molecular interaction in the solution [31].

With the change of temperature acoustical parameters also changes. Density, ultrasonic velocity and related thermo acoustical parameters of Tulsi leaf extracts solution were measured at 30°C, 35°C and 40°C. With increase of conc. of leaf extract solution adiabatic compressibility decreases, free length varies non linearly shows interaction between solute and solvent, relaxation time decreases, internal pressure decreases and free volume increases which represents hydrogen bonding [27].

2.7. Pharmacological activities of *Swertia chirayita*

i.) Anti-diabetic activity-

The most strong xanthone, swerchirin isolated from this plant extract has been proven to have great blood sugar reducing efficacy through experiments using various experimental models. Using normal and diabetic rats, the effects of structurally distinct hypoglycemic drugs, such as centpiperalone and tolbutamide have been determined [16].

Comparison of anti-diabetic activity of *Swertia chirayita* plant extract to that of the common oral medicine glibenclamide was done. The presence of mangiferin in the plant stem tissue was found to be primarily responsible for the anti-diabetic effect of plants. Clinical evaluation of *S. chirayita*'s 12% ethanolic extract and crude aqueous extract and was done in order to demonstrate the plant's ability to lower blood sugar levels through in vitro biochemical assays. In such plant crude extract, the study demonstrated the presence of primarily 3 phytochemicals, namely amarogentin, mangiferin and swertiamarin, and along with their derivatives. [32].

ii.) Antimicrobial activity-

The *Swertia Chirata* was studied in different fractions, some of which demonstrated significant anti-microbial activity at a given dose of 400 g disc -1 against the gram negative and positive bacteria and had little antifungal activity, while other fractions demonstrated mild antimicrobial activity at around 6 to 10 mm in same dose. [18].

iii.) Anticancer activity-

Amarogentin's chemopreventive and chemotherapeutic effects were assessed both continuously and after treatment in a mouse model system for liver carcinogenesis produced by carbon tetrachloride (CCl 4) and N-nitrosodiethylamine. Mice which was given amarogentin, showed no toxicity, increased body weight and improved survival. In those that had received amarogentin, there was a clear decrease in proliferation and an increase in the frequency of apoptosis [17].

iv.) Anti-Hepatitis-

On the HepG 2.2.15 cells line, all of this plant's chemical compounds were tested for anti-hepatitis B virus activities. Some of these compounds inhibited the secretion of surface antigen of hepatitis B, while others demonstrated efficacy against hepatitis B e antigen secretion (also known as HBeAg), and eight of the compounds had activity against HBV DNA replication [19].

v.) Antiviral activity-

Study shows that *S. chirayita* has ability of creating a defence against herpes simplex viruses. The impact of the crude plant extract against Herpes was confirmed by expression of HSV antigen and the indirect immunofluorescence (IFA) test in the diluted condition (1:64), where it suppressed HSV-1and plaque formation was observed at a higher level of 70% [16].

vi.) Anti-inflammatory activity-

On phytocompounds' SA-1 (oleanolic acid) and SA-4 (3-hydroxylup-12-(13)-ene-17-carboxylic acid), anti-inflammatory activity was assessed. An immediate inflammatory reaction is characterised by edoema. According to the study's findings, SA-1 and SA-4 have dose-associated anti-inflammatory effect via reducing edoema in rat paws. SA-1 and SA-4 had a strong suppressive effect on inflammatory mediators by inhibiting the production of COX enzymes at various dosages [20].

vii.) Hepatoprotective activity-

Study was done to know whether the ethanolic extracts of *S. chirayita* could provide defence against hepatotoxicity brought on by the "over - the- counter medicine" paracetamol or not. Following a paracetamol treatment, the application of plant extracts returned the values of these parameters to normal. Thus, the results of the the experiment showed that paracetamol-induced hepatotoxicity may be prevented by *S. chirayita* extracts [21].

The ability of *S. chirayita* plants extracts to guard against the acute hepatotoxicity caused by paracetamol (150 mg/kg) in Swiss albino mice was investigated. As measured using biochemical and histological criteria, oral administration of *Swertia chirayita* extract (100-200 mg/kg) provided a considerable dose associated protection against paracetamol-induced hepatotoxicity [19].

viii.) Anti- arthritic-

When *S. chirayita* extract was given orally in a range of doses, arthritic mouse joint homogenates showed a dose-dependent decrease in TNF, interleukin-1, and interferon, as well as an increase in interleukin-10. It is believed that mangiferin presence in the extract of *S. chirayita* is what causes the reduction of TNF-, IL-1, IL-6, and IFN-, as well as the elevation of IL-10, in the joint homogenates of arthritic mice [22].

ix.) Anti- helminthes-

25 mg/ml of crude aqueous extract of *Swertia chirayita* and methanolic extracts were made under for anthelmintic activity. To determine anthelmintic activity, both type of plant extracts were tested on mature live sheep Haemonchus contortus. 25 mg/ml concentration of methanolic plant extract completely restricted the movement of separated worms, and the results were totally comparable to those of levamisole, a common anthelmintic drug, which had a 0.55 mg/ml concentration [16].

CHAPTER 3

MATERIALS AND METHOD

3.1. Preparation of MS Media

Media was prepared by using Stock solutions, PGR's, Sucrose and Agar. Stock solutions (A-H) were prepared by adding the components of each stock solution in water separately. Stocks were measured one by one and added to a beaker and then PGR's i.e. IBA (3mg/L) and (Kn 1mg/L) were added in it. 30 g Sucrose was added into solution. Solution was made 900 ml by adding water and then pH was checked. Then agar was added to solution and volume was raised to 1000ml. Solution was boiled and after boiling 50-50 ml of it was put into jars. Autoclaving was done at 121°C and 15 psi. After autoclaving, jars were kept at room temperature (fig6).

Table 2. Media composition

Components	Volume
1. Stocks- (A – H)	
Stock A	100ml/L
Stock B	50ml/L
Stock C to H	10ml/L
2. PGR's	
IBA	3mg/L
Kn	1mg/L
3. Sucrose	30g/L
4. Agar-Agar	9g/L
pH= 5.6-5.7	

Fig. 6. Media prepared

3.2. Plant collection and subculturing

Plants were collected in the Plant Tissue Culture Lab, JUIT, Solan (1400 m altitude). Subculturing of micropropagated *S. chirayita* was done to increase the no. of plantlets for extracts preparation. All objects that are needed in for culturing such as foreceps, scalpel, media jars, petriplates, were placed in LAF for sterlilization 15-20 minutes before working in it. UV light was switched on. After 20 minutes switched off the UV light and then LAF floor was

cleaned. 2 jars of *S. chirayita* were taken and from them the plants were removed. Shoots were further put into the 20 media jars. Jars were labeled and put into culture room at temperature 25°C and humidity 73% (fig. 7). This was done multiple times in order to get multiple plants.

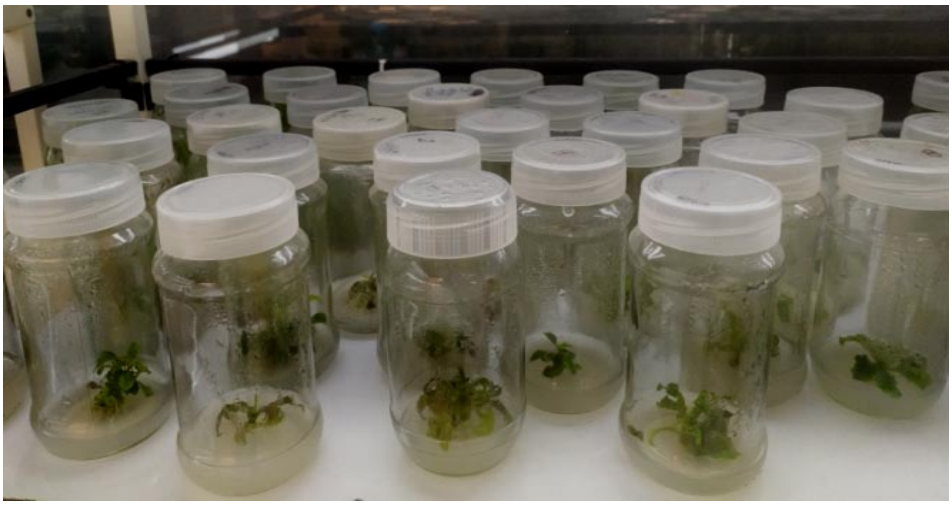


Fig.7. Subculturing of *S. chirayita*

3.3. Sound exposure to *in vitro* raised *S. chirayita*

A setup was made to expose the plants under sound to check the growth of the plants. A frequency modulator was connected to the speaker in order to get the sound of different frequencies (fig. 8). The plants were exposed under sound frequencies 500Hz (0.686 m wavelength), 1kHz (0.343 m wavelength) and 1.5 kHz (0.228) each for 4 hrs daily for 10 days in each frequency.

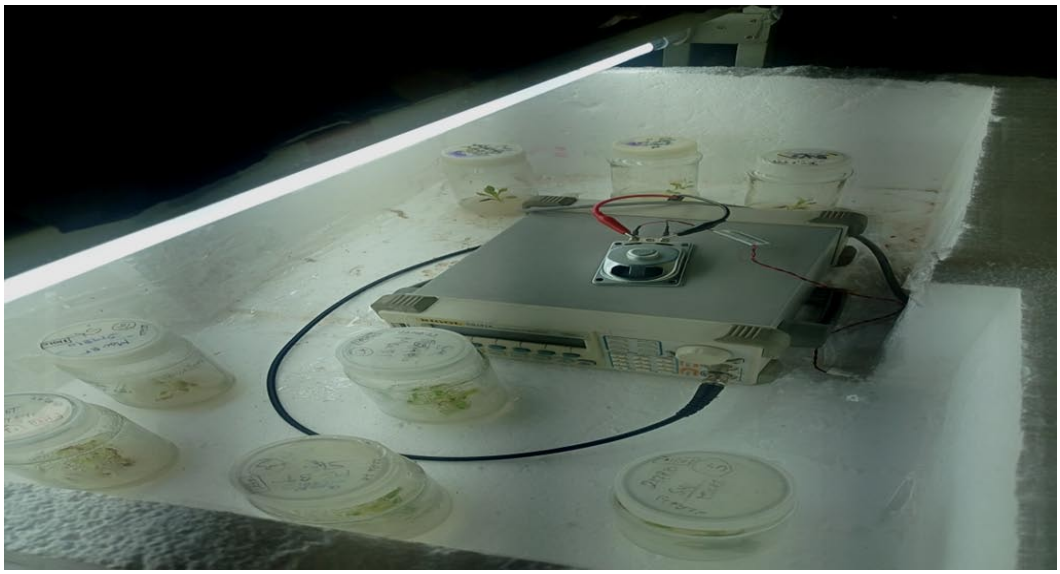


Fig.8. Plants exposed under Sound system

3.4. Extract Preparation

Extract of plants kept in culture room (control) and in sound setup with 3 diff. frequencies was prepared individually. Plants were washed and dried and then grind in mortar & pestle with the help of liquid nitrogen. Powder formed was put into 100ml of 80% methanol and kept for shaking for 24 hrs. After 24 hrs, it was filtered with the help of whatman filter paper followed by syringe filter. 4 different extracts were prepared and kept at 4°C.

3.5. Phytochemical analysis of *in vitro* raised *S. chirayita* extracts

Quantitative and qualitative analysis was done to check the presence of phytochemicals and their quantity in all 4 extracts using standard methods.

3.5.1 Qualitative analysis of *in vitro* raised *S. chirayita* extracts

i.) Alkaloids test

Mayer's test- For this test Mayer's reagent was prepared firslty in which two solutions were prepared individually i.e. 1.35g mercuric chloride (HgCl) dissoleved into 60ml distilled water and potassium iodide dissolved in 10ml distilled water. After this both of them were mixed and volume was raised to 100ml.

Then, 4 test tubes were taken and each of 4 extracts (1 of control and 3 of sound exposed plants at different frequencies i.e. 500 Hz, 1 kHz and 1.5 kHz) and were put in different test tubes. 5-6 drops of Mayer's reagent was added to that of each test tube containing extracts. Yellow ppt formation shows the postitive results [39].

ii.) Flavonoids test

Alkaline reagent test- In 4 test tubes, 2 ml of a mixture of 2.0% NaOH added to plant extract resulting yellow colour solution. After adding few drops of HCl to it, the yellow solution becomes colourless. The presence of flavonoids was demonstrated by this result [38].

iii.) Glycosides test

Killiani-Keller test- To 10 ml aqueous plant extract, 1 ml concentrated H2SO4 and 4 ml glacial acetic acid solution were added. After this a drop of a 2% FeCl3 added. The presence of cardiac steroidal glycosides was seen as a brown ring that formed between the layers [38].

iv.) Phenolic compounds test

Braymer's test- 1ml of each extract was put into 4 different test tube. Then, 1 ml of water added followed by 3 drops of 10% FeCl3 was added in each test tubes. Occurrence of deep dark blue colour appears if the tests are positive [39].

v.) Tannins test

5 ml extract was taken in 4 test tubes followed by 2 ml 5% FeCl3 solution in each test tube containing extracts. Blue, green, purple or red colour indicates positive results for tannins [39].

vi.) Terpenoids test

5 ml of each aqueous plant extract was taken in a test tube and 2.0 ml of chloroform was added in each of them. Then it was evaporated on the water bath, and then combined with 3 ml of concentrated H2SO4 to boil. Reddish brown colour on interface shows positive results [38].

vii.) Steroids test

1 ml of each 4 extracts was taken and put into 4 different test tubes. Then in each test tubes 2-3 ml chloroform was added followed by equal amount i.e 1-2 ml conc. sulfuric acid by sides of test tube to form layer. Upper layer of test tube turns red or brown and H2SO4 layer shows yellow with green colour if tests are positive [40].

3.5.2. Quantitative analysis of *in vitro* raised *S. chirayita* extracts

3.5.2.1. Total phenolic content (TPC)

50 mg Gallic acid was dissolved in 5 ml methanol (10mg/ml). Different dilutions of gallic acid were made in order to plot the standard graph of gallic acid that were dissolved in methanol.. In 4 test tubes, the 4 extracts were added. After this, water was added in all test tubes including gallic acid dilutions and 4 test tubes containing extracts. Then 100ul FC reagent was added in each test tube and kept for 6 min incubation. After incubation 1 ml Na2CO3 was added and kept for 30 min incubation. Absorbance was recorded at 760nm. Experiment was done in triplets. Total amount of phenolic content was shown in mg gallic acid and was checked by the caliberation curve $y=0.087x-0.645$, $r^2=0.9812$.

3.5.2.2. Total Flavonoids content (TFC)

50mg Quercetin was added to 5 ml methanol (10mg/ml). Different dilutions for quercetin was prepared in order to plot standard that were dissolved in methanol. To 4 test tubes, 4 different extracts were added. Then to all these test tubes 4ml water was added followed by 300ul sodium nitrate. Kept for 5 minutes incubation and then 300ul AlCl3 was added. Then after 5 minutes incubation, 200ul NaOH was added. Absorbance was recorded at 510nm. Experiment was done in triplets. Total flavonoids content was checked by the eqn. $0.127x+0.0843$, $r^2 = 0.9179$ and was shown by mg quercetin.

3.6. Antioxidant activity of *in vitro* raised *S. chirayita*

3.6.1. DPPH Free Radical Scavanging activity of *in vitro* raised *S.chirayita*

Gallic acid solution was prepared by adding 50 mg in 5 ml methanol (10mg/ml) and 0.002% DPPH (diphenyl-1-picrylhydrazyl) solution were prepared by dissolving 10 mg DPPH in 5 ml methanol. Different dilutions of quercetin were made for standard graph having conc. 50ul, 100ul, 200ul, 400ul that were dissolved in methanol with 400ul, 350ul, 300ul, 200 ul concentration respectively. 4 test tubes contains different extracts each. 3.6ml of DPPH was added to each test tube. It was kept for 30 minutes incubation and then absorbance was recorded at 517nm. Experiment was done in triplets. %RSA was calculated by following formula-

$$\% \text{ RSA} = \frac{Ab_{control} - Ab_{sample}}{Ab_{control}} \times 100$$

3.7. Acoustical Studies of *S. chirayita* extracts

Acoustical studies of all 4 extracts was done and to calculate different acoustical parameters velocity, viscosity and density in the liquid extracts is needed to check.

3.7.1. Velocity measurement

Firstly the velocity of solvents used in the extracts i.e. water and methanol was done at different temperatures to check the molecular interactions in them . Water bath was set at different temperature i.e. 20°C, 25°C, 30°C, 35°C, 40°C. Methanol and water were given these

temperatures one by one. After reaching at these temperatures, Velocity meter was Switched On and solvents were poured in liquid cell of velocity meter. Cap was closed and knob was connected. On screen, Option 1- Liquid was selected and entered. Then Option 2- Auto mode was selected and entered. All readings of velocity of solvents at diff. temperatures were checked with this procedure. Readings of velocity at different temperatures was recorded.

Fig. 9. Waterbath

Fig.10. Ultrasonic Velocity meter

3.7.2. Density measurement

Secondly, density of all 4 liquid extract was checked. It was checked with the help of RD Bottle (Relative density bottle). Weight of empty RD bottle was taken using weighing balance. The weight of RD bottle filled with water was taken. Now the weight of RD bottle filled with each extract was taken.

Then, Density of the liquid extract was checked using following formula-

Density of Extract= (Weight. of extract/Weight. of water) x Density of water

= (Weight. of extract- Weight. of empty bottle/Weight. of water-Wt of empty bottle) x Density of water

Fig.11. RD Bottles filled with water and extract

3.7.3. Viscosity measurement

Viscosity of all 4 samples were measured at different temperatures with the help of viscometer. 00 spindle was used in which the samples were put ne by one. Spindle started rotating after switching it on. Readings were recorded.

Fig.12. Viscosity measurement

3.7.4. Acoustical parameters

After checking velocity, viscosity and density, further acoustical parameters were calculated using following acoustical parameter formulas –

i. Adiabatic compressibility, $\beta a = 1/U^2\rho$

where, βa is adiabatic compressibility,
U is ultrasonic velocity
ρ is density [15]

iii. Acoustic impendence, $Z = U\rho$

where, Z is acoustic impedance
U is ultrasonic velocity
ρ is density [15]

iii. Relaxation time, $\tau = 4/3\ \eta\beta a$

where, τ is relaxation time
η is viscosity
βa is adiabatic compressibility [31]

CHAPTER 4

RESULTS

4.1. *In vitro* raised plants exposed to sound waves

This study showed the effects of acoustic waves on the growth of the plants. Plants exposed under sound system gave better results than control plants. 5 plants as control without sound exposure and 5 as tests exposed to sound from each frequency i.e. 500 Hz (Fig. 13, Table 3), 1 kHz (Fig. 14, Table 4) and 1.5 kHz (Fig. 15, Table 5) were compared after 10 days. The plants which were given the sound exposure showed fast growth and increased no. of shoots as compared to control plants. In each frequency, the length of test plants was more as compared to control plants. Shoot no. was also increased in test plants while it was same in control plants after 10 days. Although growth and development was in fast in each of the frequency but more no. of shoots and length was observed in 1 kHz. 1kHz frequency showed good results (Table 4, Fig 14,) while 1.5 kHz plants started dying after few days of exposure.

4.1.1. At 500 Hz

Table 3. Growth parameters observed at 500 Hz

Plants	Test samples				Plants	Control samples			
	Shoot length		No. of Shoots			Shoot length		No. of Shoots	
	Before	After	Before	After		Before	After	Before	After
T1	1.1 cm	1.6 cm	5	8	**C1**	1.7 cm	-	-	-
T2	0.6 cm	2 cm	4	7	**C2**	1.3 cm	1.6 cm	4	5
T3	1.4 cm	1.6 cm	4	8	**C3**	1 cm	1.3 cm	5	6
T4	1.5 cm	-	-	-	**C4**	1.2 cm	1.6 cm	5	6
T5	0.9 cm	1.3 cm	3	5	**C5**	1.1 cm	1.4 cm	3	4

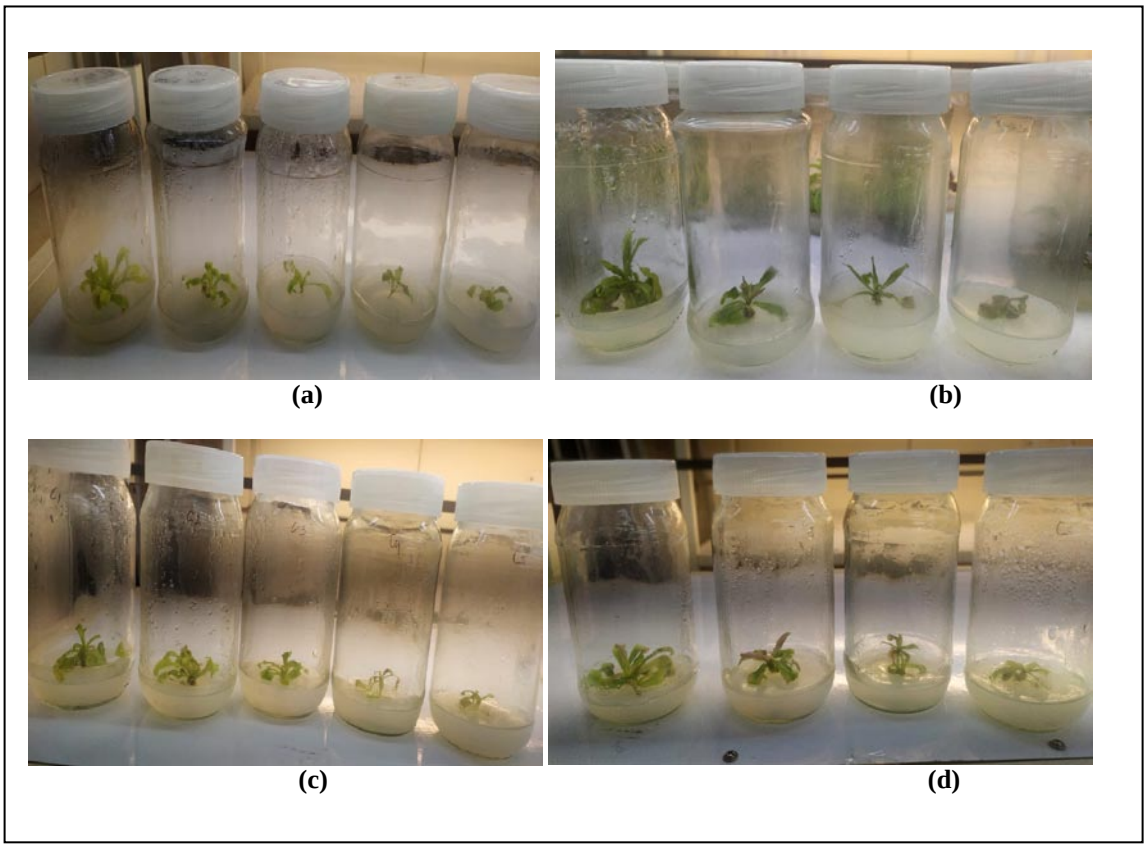
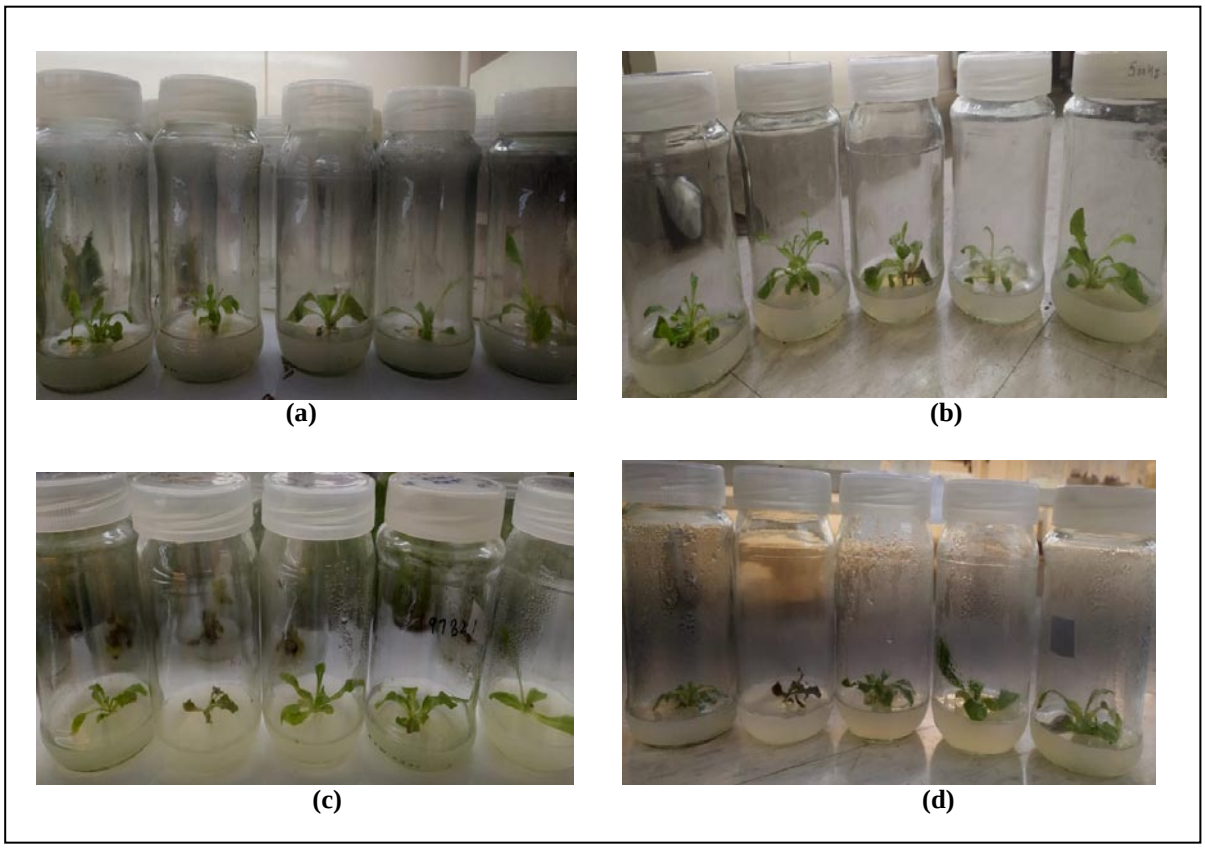


Fig. 13. Plants under the effects 500 Hz. (a) Test plants before sound exposure, (b) Test plants after 10 days sound exposure, (c) Control plants on the day of culturing, (d) Control plants after 10 days of incubation

4.1.2. At 1 kHz

Table 4. Growth parameters observed at 1 kHz

Plants	Test samples				Plants	Control samples			
	Shoot length		No. of Shoots			Shoot length		No. of Shoots	
	Before	After	Before	After		Before	After	Before	After
T1	1.2 cm	1.8 cm	3	5	**C1**	1.1 cm	1.3 cm	4	4
T2	1.8 cm	2.3 cm	4	6	**C2**	0.9 cm	1 cm	3	3
T3	1.4 cm	1.8 cm	4	5	**C3**	1.3 cm	1.5 cm	4	5
T4	1.3 cm	1.9 cm	4	6	**C4**	0.6 cm	0.9 cm	4	4
T5	1.6 cm	2.1 cm	3	5	**C5**	0.8 cm	1.1 cm	4	4



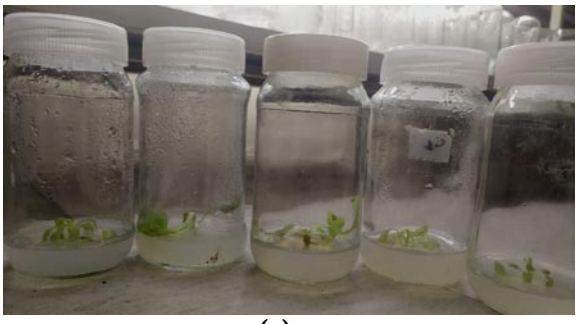
(a) **(b)** **(c)** **(d)**

Fig.14. Plants under the effects of 1 kHz. (a)Test plants before sound exposure, (b) Test plants after sound exposure for 10 days, (c) Control plants on the day of culturing, (d) Control plants after 10 days of incubation

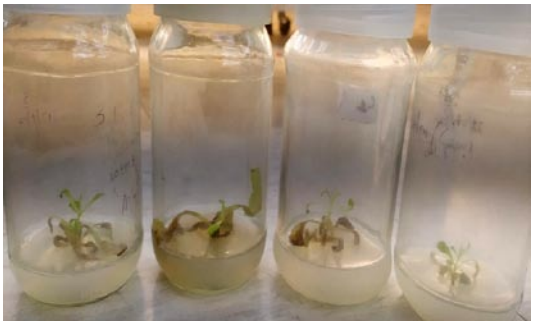
4.1.3. At 1.5 kHz

Table 5. Growth parameters observed at 1.5 kHz

Plants	Test samples				Plants	Control samples			
	Shoot length		No. of Shoots			Shoot length		No. of Shoots	
	Before	After	Before	After		Before	After	Before	After
T1	1.4 cm	2 cm	4	6	**C1**	1 cm	1.4 cm	4	5
T2	1 cm	-	-	-	**C2**	1 cm	-	-	-
T3	0.5 cm	1.2 cm	3	6	**C3**	0.3 cm	0.8 cm	4	4
T4	1 cm	2.2 cm	4	6	**C4**	1.4 cm	1.9 cm	5	6
T5	0.9 cm	1.6 cm	5	6	**C5**	0.7 cm	1 cm	4	4



(a)



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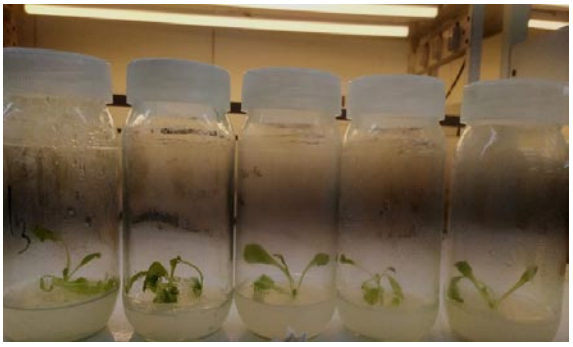
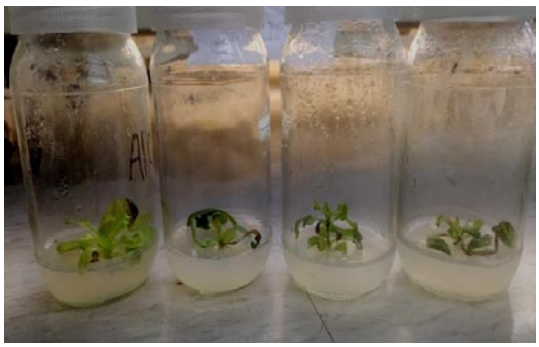


(c)



(d)

Fig. 15. Plants under the effects of 1.5 kHz. (a) Test plants before sound exposure, (b) Test plants after 10 days sound exposure, (c) Control plants on the day of culturing, (d) Control plants after 10 days incubation

4.2. Phytochemical analysis of *in vitro* raised *S. chirayita*

4.2.1. Qualitative tests

In this study, various phytochemical analysis were done to check the presence of them in plant. Control plant and test plants having 500Hz, 1kHz and 1.5 kHz were tested for qualitative phytochemical tests. Tests for tannins, phenols, terpenoids, flavonoids, glycosides, steroids, and alkaloids were done in which tests for phenols, steroids, flavonoids, and tannins were positive that shows presence of them in samples while alkaloids, terpenoids and glycosides test showed negative results in each sample as shown in Table 6.

Table 6. Qualitative phytochemical analysis

Phytochemicals Tests	Control plants	Sound exposed plant (500 Hz)	Sound exposed plant (1 kHz)	Sound exposed plant (1.5 kHz)
Alkaloids	-	-	-	-
Flavonoids	+	+	+	+
Glycosides	-	-	-	-
Phenols	+	+	+	+
Tannins	+	+	+	+
Terpenoids	-	-	-	-
Steroids	+	+	+	+

4.2.2. Quantitative tests

4.2.2.1. Total Phenolic Content (TPC)

This study showed that sound waves affects the Total Phenolic Content (TPC) of plants. Total phenolic content was compared between the sound exposed plants and control plants. The plants which were exposed to sound increased the TPC of plants which varies from 1.315 to 1.486 mg GA g^{-1}. Maximum accumulation of total phenolic content i.e. 1486 mg GA g^{-1} was observed in 1kHz while minimum accumulation was in 500 Hz frequency i.e. 1.315 mg GA g^{-1}. Plants exposed to 1kHz and 1.5 kHz accumulated more phenolic content as compared to control plants, but 500 Hz plant showed less total phenolic content than control plants as shown in Fig. 16.

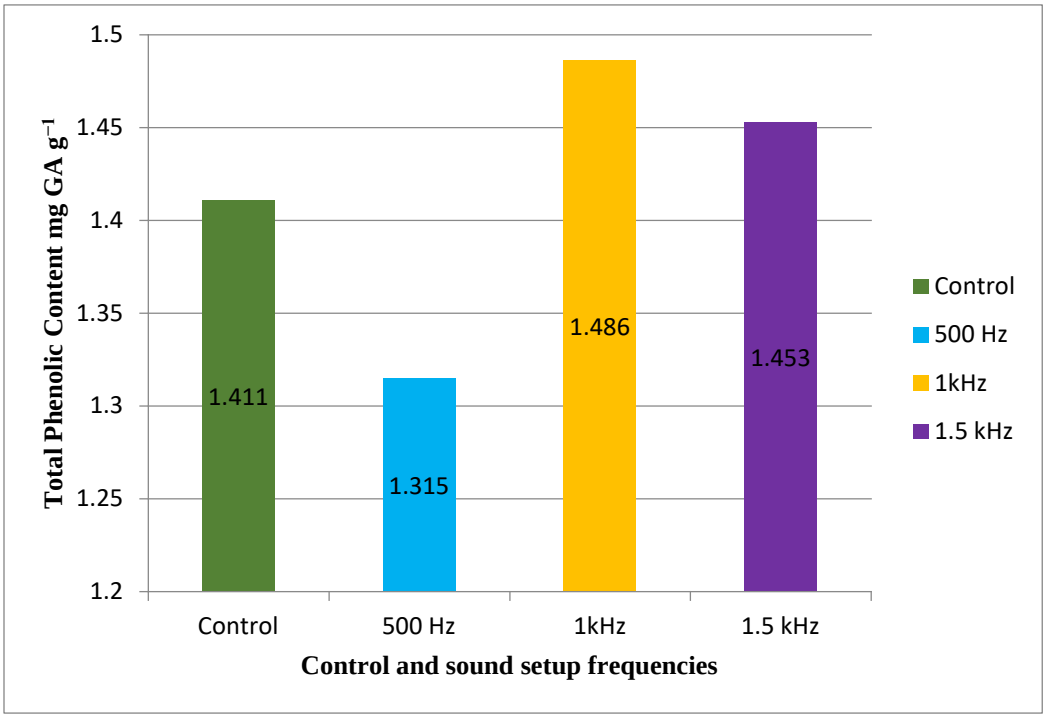


Fig. 16. Impact of sound waves at different frequencies on the total phenolic content of *Swertia chirayita* after 10 days

4.2.2.2. Total Flavonoid Content (TFC)

According to this study, sound waves affected the Total Flavonoids Content in *S. chirayita*. TPC was increased in the sound exposed plants. It was checked by comparing flavonoids content of contol plant to flavonoids content of test plants. The flavonoids content range varies from 0.106 to 0.19 mg mg QE g^{-1} . Total flavonoids content in control was 0.106 mg QE g^{-1} which was less than sound exposed plants. Highest amount of flavonoids among sound exposed plants was accumulated by 1kHz frequency plants, i.e. 0.19 mg QE g^{-1} while least amount was accumulated by 500 kHz, i.e. 0.14 mg QE g^{-1} (Fig. 17) . Control plant accumulated less flavonoids content as compared to test plants.

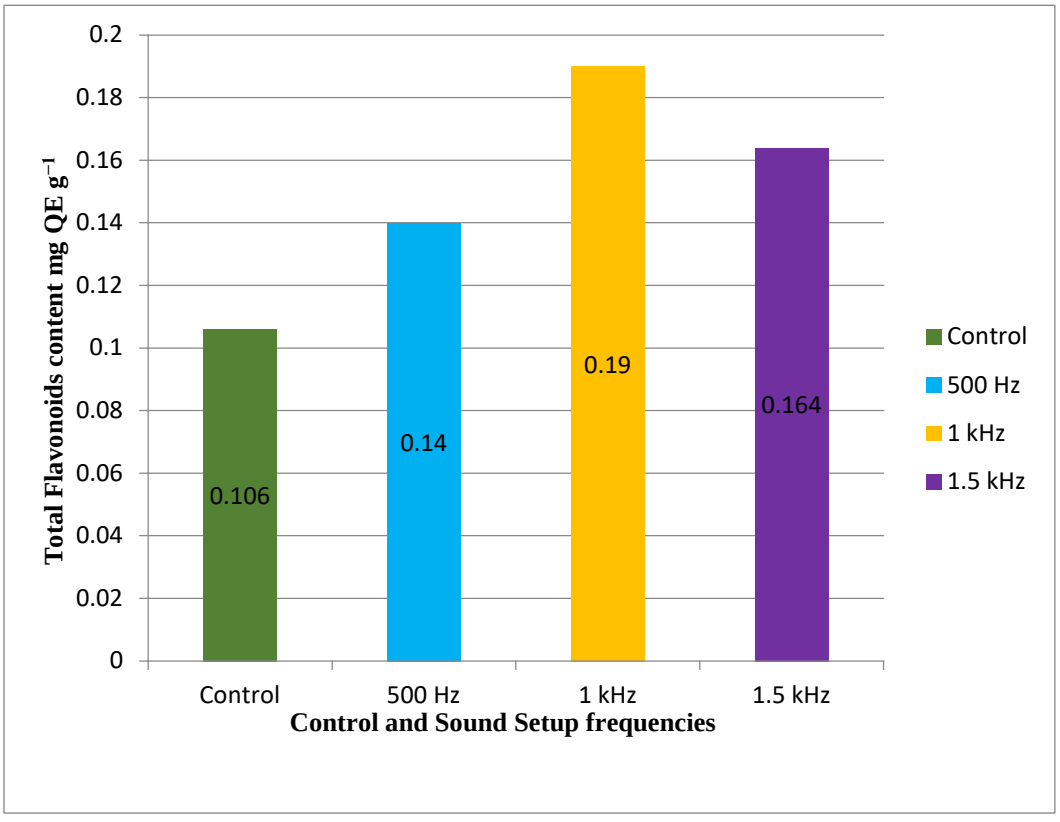


Fig. 17. Impact of sound waves at different frequencies on the total flavonoids content (TFC) of *Swertia chirayita* after 10 days

4.3. DPPH assay

The antioxidant activity was checked by DPPH assay in each sample. This study shows that sound waves affected the % radical scavenging activity in the test plants. The control sample has the less percentage of RSA while it was in much amount in the test samples. In control samples it was only 47.39% while in plants exposed to 500 Hz it was 54.40%, 1 kHz it was 64.68% and 1.5 kHz it was 57.75%. The highest percent inhibition or percent RSA among sound exposed plants was observed in 1kHz plant i.e. 64.68% and least was in 500kHz plants i.e. 54.40% as shown in Fig. 18.

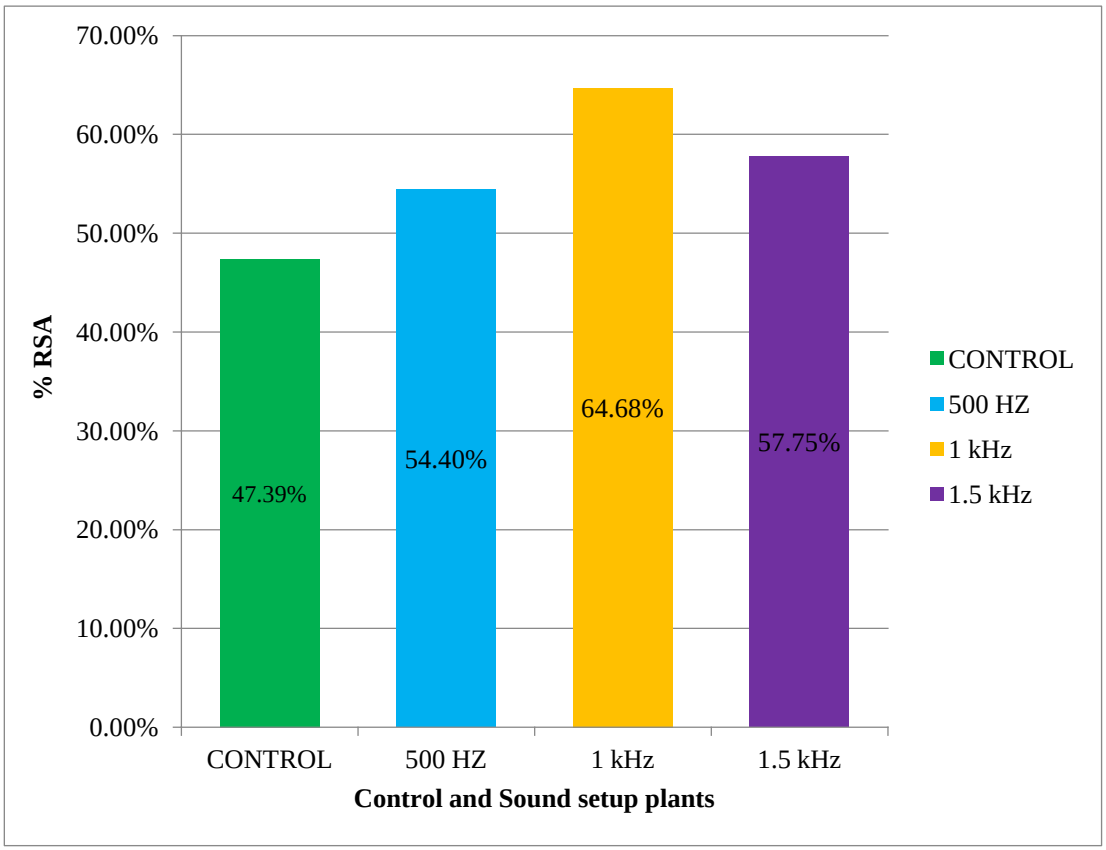


Fig. 18. Impact of sound waves at different frequencies on the % Radical Scavenging Activity of *Swertia chirayita* after 10 days

4.4. Acoustical study of *in vitro* raised *S. chirayita* extracts

Table 7. Acoustical parameters of *S. chirayita* extracts

Acoustical Parameters	Temperature K	Plants			
		Control	500Hz	1 kHz	1.5 kHz
Ultrasonic velocity, *U* *m/s*	20°C	1347.06	1446.02	1745.50	1567.09
	25°C	1330.98	1431.24	1736.21	1553.21
	30°C	1317.95	1428.67	1724.67	1540.89
	35°C	1314.20	1417.89	1711.13	1523.23
	40°C	1288.01	1401.11	1701.05	1512.90
Density, ρ Kg/m^3	20°C	920.5	948.5	1054.3	952.5
	25°C	910.9	936.1	1024.8	941.7
	30°C	898.4	930.9	1001.5	934.7
	35°C	881.8	921.6	991.4	925.3
	40°C	875.9	912.6	978.2	908.2
Viscosity, η NSm^-2	20°C	1.7012	1.8561	1.9134	1.783
	25°C	1.6023	1.8103	1.9001	1.771
	30°C	1.5441	1.7941	1.8821	1.698
	35°C	1.5022	1.7461	1.8644	1.621
	40°C	1.4811	1.7112	1.8210	1.579
Adiabatic compressibility, βa x 10^-10	20°C	5.9869	5.0421	3.1131	4.2751
	25°C	6.1970	5.2146	3.2371	4.4017
	30°C	6.4080	5.2629	3.3568	4.5059
	35°C	6.5660	5.3972	3.4449	4.6578
	40°C	6.8818	5.5818	3.5329	4.8105
Acoustic impedance, *Z x 10^4*	20°C	123.9	137.1	184.0	149.2
	25°C	121.2	133.9	177.9	146.2
	30°C	118.4	132.9	172.7	144.0
	35°C	115.1	130.6	169.6	140.9
	40°C	112.8	127.8	166.3	137.4
Relaxation time, τ	20°C	13.57	12.47	8.57	10.20
	25°C	13.23	12.41	8.42	10.16
	30°C	13.19	12.36	8.20	10.12
	35°C	13.15	12.30	8.12	10.08
	40°C	13.06	12.23	8.04	10.01

Readings of velocity, viscosity and density were taken through which further calculations were done to find the values of adiabatic compressibility, relaxation time and acoustic impedance of each sample at each temperature (Table 7). In this study, in each samples (control, 500Hz, 1kHz, 1.5kHz) it is observed that with the increase in temperature , ultrasonic velocity, density and viscosity is also decreased due to the reduction in cohesive forces between the molecules. Due to this there is a volume expansion and increased kinetic energy resulting decrease in density and velocity. It means there is less interactions between the molecules of extracts. Adiabatic

compressibility is increased with the increase in temperature which shows less interaction between molecules because adiabatic compressibility shows association and dissociation among the molecules. Acoustic impendance due to weak interaction in molecules and relaxation time due to increase in excitation energy decreases with the increase in temperature. This study shows that there is a weak intermolecular interactions in the extracts solution.

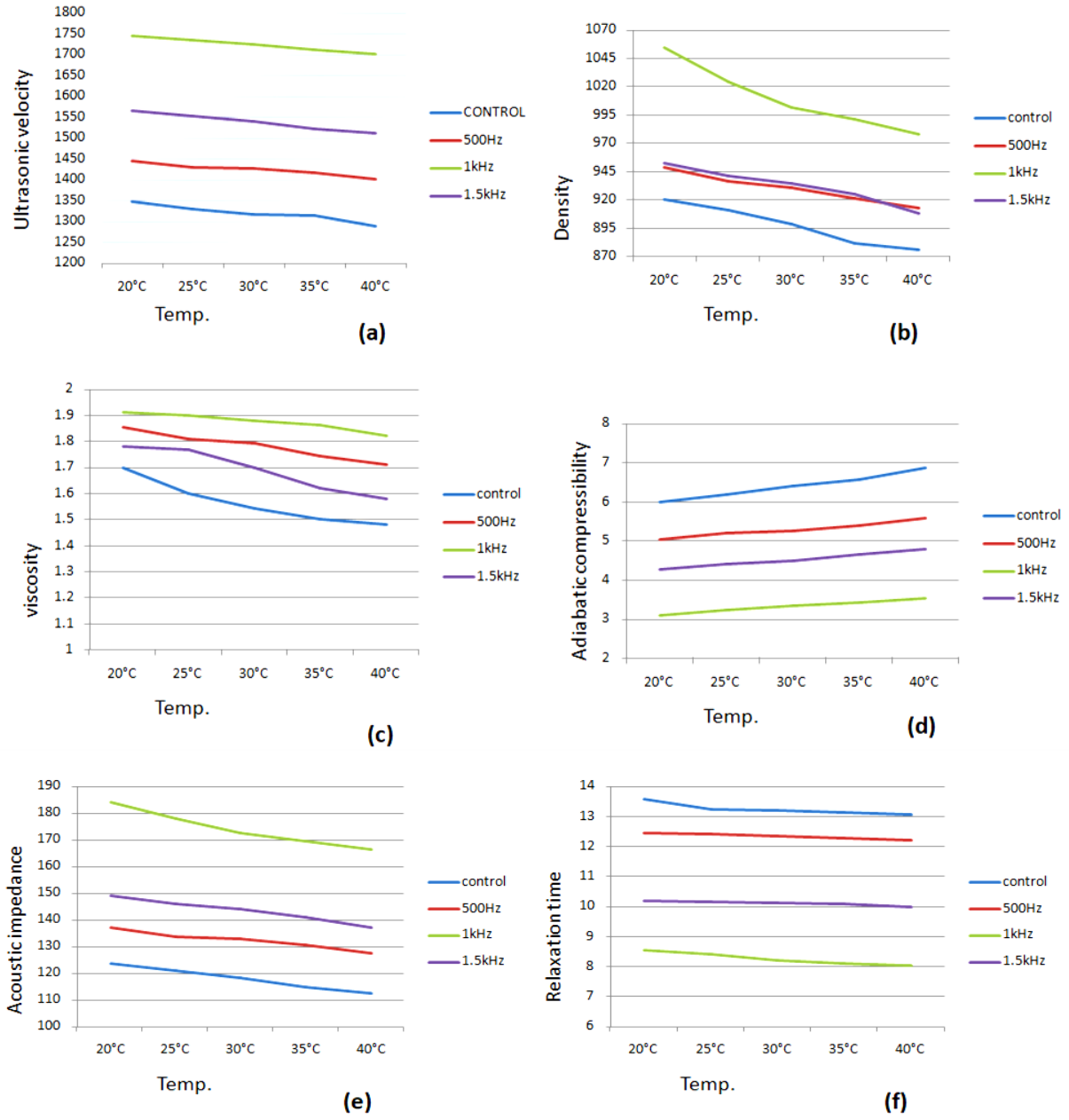


Fig. 19. Acoustical studies of *S. chirayita* extracts. (a) Ultrasonic velocity decreasing with increase in temperature, (b) Density is decreasing with increase in temperature, (c) Viscosity is decreasing with increasing in temperature , (d) Adiabatic compressibility increasing with increase in temperature, (e) Acoustic impedance decreasing with increase in temperature, (f) Relaxation time decreasing with increase in temperature

CHAPTER 5

DISCUSSION

Swertia chirayita , a medicinal herb contains a large no. of pharmaceutical values that makes this plant very important in field of research. Because it is critically endangered plants there are various researches going on to preserve this plant or to use its bioactive compounds for various uses. Acoustic waves exposure to plants can be helpful for this as reports says that they help the plants to grow faster. Present study showed that there is a positive effects of acoustic waves on the growth and development of *S. chirayita* shoots. Those waves helped the plant to grow faster as when it enters the plants it triggers the transcriptional process of plants producing the two main genes involved in photosynthesis i.e. rubisco small sub-unit (rbcS) and fructose 1,6-bisphosphate aldolase (ald). By continuous exposure of plants to sound CO2 fixation is also enhanced due to which more faster the growth will be. Thus *S. chirayita* were exposed to different frequencies i.e. 500 Hz, 1kHz and 1.5 kHz among which the 1kHz exposed plants gave better results than others. All plants grown under these frequencies grew faster than the plants kept in control. The shoot no. were also increased in the test plants [42].

As plants gave good results under acoustic waves exposure, their molecular interaction can also be checked by preparing their extracts because as their growth increased, their content is also increased that can be known by measurement of density, viscosity velocity and other acoustical parameters. Studying molecular interactions can be helpful during the drug formulation. Whatever the formulation is, various parameters are checked when it is being prepared. So affect of temperature was checked on the extracts of *S. chirayita.* So in this study it was observed that in each extract there were weak molecular interactions because with increase in temperature velocity, density, viscosity, acoustic impedance and relaxation time decreases due to weak intermolecular interactions and increase in excitation energy while adiabatic compressibility increases [31].

Qualitative and quantitative analysis of phytochemicals in all plants i.e. control and tests were done in *S. chirayita* in this study. Qualitative Tests for tannins, alkaloids, terpenoids, phenols, flavonoids, steroids and glycosides. Tests were positive only for tannins, phenols, flavonoids and steroids that shows the presence of them in *S. chirayita.* Effects of acoustics waves were

significantly seen on the TPC and TFC of all control and test plants. High amount of Total phenolic content was observed on test plants exposed to 1kHz i.e. 1.486 mg GA g^{-1} and 1.5kHz i.e. 1.453 mg GA g^{-1} than control i.e. 1.411 mg GA g^{-1} while in 500Hz plant it remain less i.e. 1.315mg GA g^{-1}. Total flavonoids content in test plants was also more than control plants. It was observed that Test plants exposed to 500Hz, 1kHz and 1.5 kHz accumulated 0.14mg QE g^{-1}, 0.19mg QE g^{-1} and 0.164 mg QE g^{-1} TFC respectively, while in control plant it was 0.106 mg QE g^{-1}. Thus it was significantly seen that TFC was increased in the sounds exposed plants than control plants [23].

Antioxidant activity was checked by % RSA values of each plant sample and it was observed that it was much in 1kHz exposed plant i.e. 64.68%. Among test plants, %RSA was higher in 1kHz plant while less in 500Hz plants i.e. 54.40%. At 1.5 kHz it was 57.75% while in control it was 47.39%. Thus it can be said that plants which were exposed to 1kHz gave best results for growth, TPC, TFC and antioxidant activity as their quantity were more than others in 1kHz [41].

CHAPTER 6

CONCLUSION

Acoustic waves showed significant results in the plant growth, development and its phytochemical constituents. By exposing plants under sound setup, they grew faster than the control plants. Plants exposed under sound accumulated more phenolic and flavonoids contents than control plants. Antioxidant activity in sound exposed plants was also more than unexposed plants. But highest amount of total phenolic content, total flavonoids contents and antioxidants activity was in plants which were exposed to 1kHz frequency. Acoustical parameters were also checked in the plants to know about the solute solvent interaction in the liquid extracts of *S. chirayita.* With the increase in temperature, there was decrease in velocity, density, viscosity, acoustic impedance, relaxation time but increase in adiabatic compressibility. These acoustical parameters are helpful during drug formulation preparation because the molecular interactions in the liquid extracts can be known.

CHAPTER 7

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