Phytochemical characterization and the therapeutic efficacy of *Fraxinus americana*

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Abstract

Fraxinus americana, a species commonly known as Ash tree is native to Eastern and Central North America and North Asia. This plant is known to posses a cluster of therapeutic properties. It is being used since ages in Unani and Ayurvedic medicine. Many infertility issues and gynecological problems can be treated by natural herbs and their extracts, Fraxinus is one such example. Objective of the current research is to characterize the various biochemical components present in the mother tincture of Fraxinus americana and testing its efficacy in treating uterine fibroids based on several biochemical and molecular assays.

Phytochemical analysis based on biochemical study was performed followed by TLC analysis. TLC would provide an insight to the array of components present in tincture along with the information about the suitable solvents for their separation. MTT assay can be used to test the efficacy of the sample in controlling cell division (anti cancer). Use of uterine fibroid related cell lines (Human Uterine Leiomyoma cell culture) would identify its role in this condition. In addition to anti cancerous activity by MTT, various other tests like anti inflammatory and antioxidant activity would be performed to annotate the complete therapeutic potential of Fraxinus americana. The study can lay a foundation for a green therapy eliminating the need for surgical procedures in treating several uterine problems.

Keywords: Anti inflammatory, Antioxidant, Fraxinus americana, Infertility, MTT assay, TLC.

Introduction

Natural herbs and their extracts are always known to be miraculous in treating several disorders and metabolic abnormalities. Natural products obtained from plants have been used in manufacture of numerous chemicals and biological products[21]. In some of the major health hazards like cancer, diabetes, obesity etc. natural plant based medicines provide a better treatment efficacy compared to synthetic and chemical products[8,14]. Tulsi *(Ocimum tenuiflorum)*, Neem *(Azadirachta indica)*, Turmeric (Curcuma longa), Cinnamon *(Cinnamomum verum)* etc. are several herbs with multi factorial functions[7]. *Fraxinus americana* commonly known as Ash tree is one among such medicinal plants known since ages for its key role in treating female gynecological problems[15]. This plant is also known for its influence in treating conditions like anxiety and depression[16]. This plant is known to possess versatile pharmacological functions.

Apart from its therapeutic actions, it is also used in cosmetic industries, in the preparation of anti ageing products etc.[18] Several biochemical components present in plant can be tested based on some standard procedures. Biochemical composition of any plant is an insight to its numerous biological functions. The current work includes biochemical characterization of the mother tincture of *Fraxinus americana* and testing its role against uterine fibroid. With high efficacy and no side effects of herbal therapies in comparison to the other chemical drugs, the current work aims to analyze the phytochemical composition and therapeutic role of this natural herb.

Material and Methods

Collection of sample: Mother tincture of *Fraxinus americana* obtained from a local medical store is a product of Dr Willmar Schwabe, India and is used as study sample. Tincture is an ethanol based extract of Fraxinus leaves. As this is most commonly used form in traditional medication, it is selected rather than leaves or fresh extract.

Preparation of standard concentration for analysis: In order to use the sample for study, a standard concentration of the tincture has to be prepared. For this, mother tincture is collected into a pre weighted sterile centrifuge tube and is allowed to dry overnight in an incubator. The dry pellet is weighed and dissolved in ethanol to obtain a final concentration of 1mg/mL. This is used in further analysis.

Phytochemical characterization: Plant material is known to possess several phytochemicals which include flavonoid, alkaloids, phenol's, terpenoids etc. Identification of alkaloids can be done by adding 1% HCl to the extract[3]. Solution must be heated with constant stirring for about 15 to 20 min followed by cooling. To the extract, few drops of Wanger's reagent should be added. Presence of alkaloids is observed by the formation of creamy brown solution. Alkaline reagent test is a standard procedure for flavonoid detection which involves addition of 2-3 drops of NaOH to the extract which forms a yellow coloration which disappears upon the addition of dilute HCl[12].

Test for the identification of tannins includes initial heating of 2ml extract followed by the addition of 3ml ferric chloride. Orange coloration is a confirmation for the presence of tannins in the extract[4]. Phenols can be tested by

adding 5ml distilled water to 3ml of extract. Formation of dark green color complex upon addition of 5% ferric chloride confirms the presence of phenols[1]. Test for saponins can be performed by adding 2ml water to 3ml of extract followed by vigorous shaking. Formation and persistence of froth are indication of saponins in the extract[13]. Extracts are first treated with HCl followed by the addition of sodium nitroprusside in pyridine and sodium hydroxide[2]. Presence of cardiac glycosides is indicated by the formation of pink to red complex.

Terpenoids are aromatic components in plants responsible to maintain normal growth and activity. The presence of terpenoids in the extract can be tested by Salkowski test which involves mixing the test sample with chloroform and concentrated sulphuric acid to form a layer[6]. Reddish brown colour will develop at the junction of solution indicating the presence of terpenoids.

Test for Coumarins: Coumarins are tested by adding 10% NaOH to test sample which changes the colour to yellow confirming the presence of coumarins[19].

TLC separation of compounds: Thin layer chromatography involves the separation of compounds on a stationary phase using the capillary effect of mobile phase running across it. All the individual components are separated as bands on the TLC plate based on their retardation factor, which is specific for a given compound and mobile phase combination. Once all the compounds are separated, they are visualized either under visible light or UV light to observe various bands corresponding to different compounds. Compounds in the bands can be identified based on the retardation factor RF values.

Antioxidant activity assay: Protection of cells from the free radicals and their toxic effects is possible by antioxidants. This property is also considered as one of the key factors in anti ageing. Several external agents like fruit juices and plant extracts act as natural suppliers of antioxidants to the body. Antioxidants would also provide anti-cancerous potential to the body which is a major advantage of these compounds. Presence of antioxidants in the plant extracts can be tested by several methods among which DPPH assay and FRAP assay are most prominent.

DPPH Radical scavenging assay: Free radical scavenging activity of test sample (tincture) was detected based on DPPH (1,1-diphenyl-2-picryl hydrazyle) assay[20]. For the test, 24mg of DPPH should be dissolved in 100ml methanol which forms the stock solution. This mixture can be filtered and used for detection of absorbance at 517nm. This sample reading can be considered as control. Test sample can be prepared by adding various dilutions of tincture with DPPH. All the tubes were incubated in dark at room temperature for 20minutes and absorption is read at 517nm. Radical scavenging activity of the sample can be calculated using the formula

$$\%\text{Radical Scavenging activity} = \frac{\text{OD of Control-OD of sample}*100}{\text{OD of control}}$$

A plot of % radical scavenging activity against concentration of test sample is needed to obtain the IC_{50} value for the test sample. IC_{50} can be calculated using the linear regression curve.

FRAP assay: Ferric reducing antioxidant power assay following the procedure of Benzie and Strain was performed[9]. The test involves reduction of Fe^{+3} TPTZ a colourless complex to a blue coloured Fe^{+2} Tripyridyltriazine complex. This is due to the electrons donated by the oxidizing agent to the test sample under acidic conditions. The change in the absorption is measured at 593nm. FRAP reagent can be prepared by the addition of 300mM acetate buffer to 10mM TPTZ. To this, 40mM HCl and $FeCl_3.6H_2O$ must be added. All the components are added in the ratio of 10:1:1 at 37^0C.

To freshly prepared 3.995ml of FRAP, 5µl of test sample must be added. Intense blue colour complex will be formed whose absorption is read at 593nm. Blank containing 3.995ml of FRAP reagent with 5µl water is used with an incubation at 37^0C for 30min. Readings are taken in triplicate to minimize the error. A standard calibration curve should be plotted for various concentrations of $FeSO_4$. Absorption values of the sample are compared to those of standard FRAP using a plot.

Testing the anti inflammatory activity: Anti inflammatory activity was tested based on the principle of proteinase inhibition test proposed by Sakat et al and modified by Gunathilake et al[10]. A solution containing 0.06mg of trypsin in 1ml of 20mM tris HCl is added to 1ml test sample. The solution was incubated at 37^0C for 5min. To the above solution, 1ml of 0.8% casein was added and incubated for 20min. After incubation, 2ml of 70% perchloric acid was added to terminate the reaction. Solution must be centrifuged to collect the supernatent whose absorbance can be recorded at 210nm using buffer as a blank. Inhibition percentage % of denaturation for protein is calculated as follows:

% inhibition of protein denaturation= 100 * 1-A2/A1.

where A1= absorption of control and A2= absorption of test sample.

MTT assay of extract against Human Uterine Leiomyoma cell culture: Cytotoxicity of mother tincture on uterine leiomyoma cell culture was tested by a colorimetric assay using dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[5]. The reaction involves the detection of viable cells based on the reaction of NADPH of these cells with tetrazolium rings forming violet formazan crystals. Solubilization of crystals is performed in DMSO. The intensity of color is proportional to the number of viable cells

present in the well which is read at 540nm using an Elisa plate reader. Wells are seeded with 180µl of media containing cells in a range of 1×10^4 density per well. Once the seeded plate was ready after 24hrs incubation, mother tincture was loaded in different concentrations 10, 20, 30µg/ml in triplicate, leaving the first two rows without extract, which can be considered as blank.

Plate was incubated for 48hrs at 37^0C and 5% CO_2. To all the wells including blank, 20µl of MTT solution in phosphate buffer at a concentration of 5mg/ml was added and re-incubated for 3hrs. Later the old medium was discarded from the wells and replaced with DMSO to solubilize the Formazan crystals formed. Absorbance of each well was determined at 540n in an Elisa plate reader. Standard graph was plotted containing absorbance against the cell no. Viability of cells was calculated based on the standard plot. In the initial 2 rows considered as blank, % of cell viability was considered to be 100.

Results and Discussion

Current work aimed to test and confirm the biological activities and benefits of *Fraxinus americana* whose mother tincture is used in treating several ailments. Commercially available *Fraxinus americana* mother tincture was used for the study. Use of mother tincture is preferred over manual extraction as the mother tincture is routinely used for direct oral intake and its study can be more practically applicable. The tincture was subjected for basic biochemical testing for various components. All the results are furnished in table 1.

From the results of phytochemical screening table 1, it can be observed that *Fraxinus americana* mother tincture contained the following components which include tannins, steroids, alkaloids, flavonoids, phenols and glycosides. However, the concentration of these compounds may vary. Saponins were not detected in sample.

DPPH assay for estimating the antioxidant activity: Results of DPPH assay are summarized in table 2. Results in table 2 depict a direct relation between concentration of sample and antioxidant activity observed. Higher is the concentration of extract, higher is the antioxidant activity. IC 50 value obtained for the sample was 5.288 which is concentration dependant. Redical scavanging activity of the samples is analyzed and the results are summarized in figure 1.

Table 1
An overview of Phytochemical tests performed and the constituents identified in Mother Tincture

Component	Presence
Alkaloid	+
Flavonoid	+
Tannin	+
Steroids	+
Phenol	+
Saponins	-
Glycosides	++
Coumarins	+

Based on the plot seen in figure 1, R2 Value was calculated to be 0.9413 and the linear regression equation is specified above. There exists an inverse relation between antioxidant activity and IC50 value. Higher IC50 is an indication of low antioxidant activity of the sample. This can be compared to the standard antioxidants like ascorbic acid.

Compound separation on TLC: Different combinations of mobile phases n-hexane and ethyl acetate were used to get good separation of compounds from mother tincture and the results obtained were shown in figure 2.

Figure 2 shows the separation of different compounds present in *Fraxinus americana* mother tincture on TLC plate observed under UV transilluminator. All the six plates correspond to 3 sets of mobile phases which include n hexane and ethyl acetate in the ratios of 13:7, 5:5 and 8:2 respectively. In each set of TLC plates, the first one corresponds to single spotting of sample and the second one corresponds to multiple spotting to minimize sample loading error.

Table 2
Details of absorption values obtained for various concentrations of sample in DPPH assay

S.N.	Sample	Absorbance at 517nm	% RSA	IC 50 Value
1	Blank (Control)	0.900	-	-
2	Test 1 (25 µg/mL)	0.670	25.5	0.653999444
3	Test 2 (50 µg/mL)	0.500	44.4	2.971174344
4	Test 3 (75 µg/mL)	0.450	50	5.288349245
5	Test 4 (100µg/mL)	0.363	59.66	7.605524145

Based on the above results, the mobile phase with 50% n hexane and 50% ethyl acetate is found to be good in obtaining better compound separation. RF values were calculated for the above bands and the compounds showing prominent bands. Standard RF values of Fraxin and Fraxetin in hexane and ethyl acetate were 0.01 and 0.05 respectively as observed in literature[17].

Testing the antioxidant activity of sample by FRAP assay: Antioxidant activity imparts a good anti cancerous nature of any of the sample. The antioxidant capacity of tincture was tested using FRAP reagent. Results are studied on calibration curve detailed in figure 3.

The graph shown in figure 3 displays and compares the antioxidant activity of mother tincture with standard $FeSO_4$. The results show a proportional relation between the antioxidant activity and concentration. The data furnished here is for standard ($FeSO_4 \cdot 7H_2O$: 0.1–1.5 mM in methanol) and test sample in 2 replicas for accuracy of results.

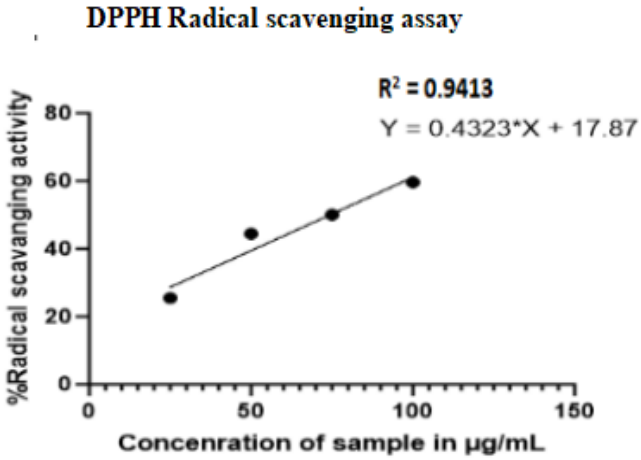

Figure 1: Plot showing the % Radical scavenging activity of sample at various concentrations in µg/mL

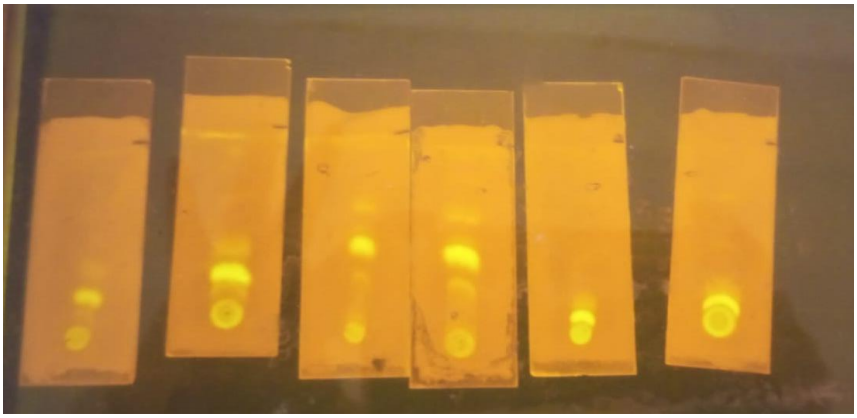

Fig 2: Various TLC plates developed in different ratios of mobile phases (n Hexane and Ethyl acetate). Bands were observed under UV.

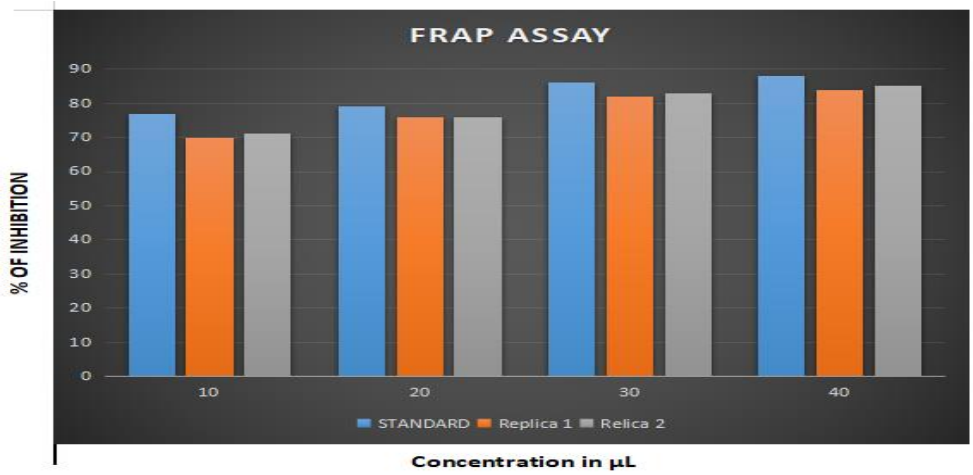

Figure 3: Plot showing the antioxidant activity of test sample based on its comparison with the standard FRAP

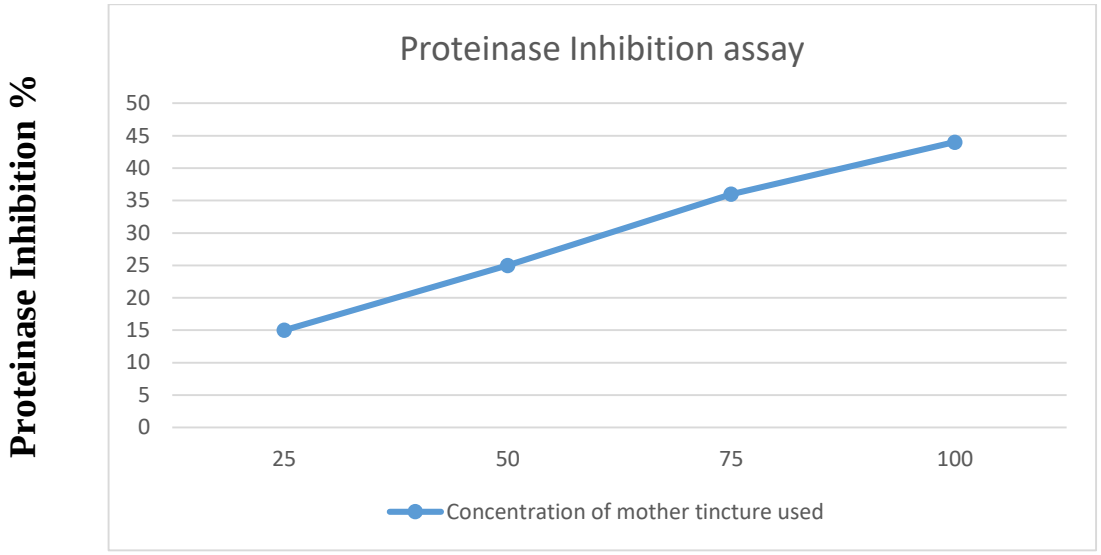

Figure 4: Plot showing the Anti inflammatory activity of sample based on proteinase inhibition assay

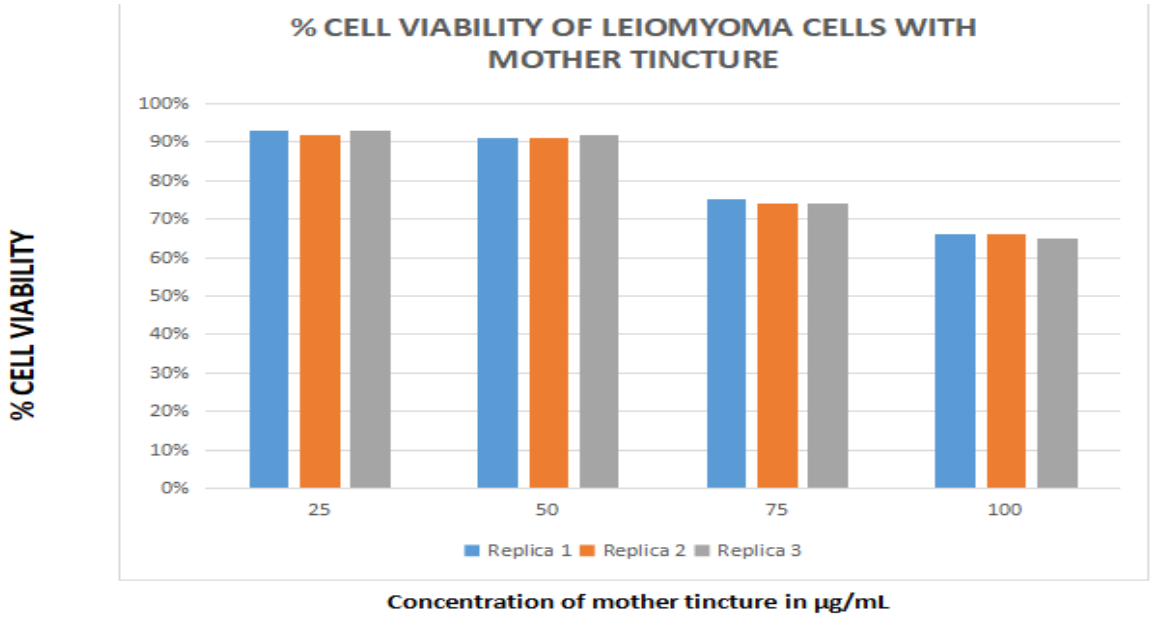

Figure 5: Showing the decreasing cell viability

Plot shows the decrease in the cell viability with an increasing concentration of mother tincture used. This successfully proves the inhibitory activity of the extract against fibroids.

Figure 4 depicts the relation between concentration of tincture and its proteinase inhibition percentage. There is a linear relationship indicating a good anti inflammatory activity of *Fraxinus americana* mother tincture. Percentage of proteinase inhibition is plotted against concentration of extract in µg/mL.

Testing the anticancer activity of mother tincture by MTT assay on Human Uterine leiomyoma cell cultures: MTT assay identifies the role of a test compound on metabolism and cell viability. Study involved the use of uterine leiomyoma cell cultures to detect the inhibitory activity of mother tincture on leiomyoma cell viability. Based on the role of the extract, the cells will show either no response or decreased metabolic activity or cell death. The effect is to be identified based on the development of color which is read using a Elisa reader. The results are furnished as a graph in figure 5. Plot in figure 5 shows the decrease in the cell viability with an increasing concentration of mother tincture used. This successfully proves the inhibitory activity of the extract against fibroids.

The results shown in figure 5 graph reveal the effective control of mother tincture on cell division rate indicating its best applicability in cancer therapy. Decrease in cell viability was directly proportional to concentration of mother tincture indicating its efficacy in cancer control.

Conclusion

The current study aimed for a detailed biochemical characterization of *Fraxinus americana* mother tincture to understand its role in treating uterine leiomyoma's. The

work involves testing of biochemical properties of *Fraxinus americana* mother tincture which is readily available in medial stores and is generally used in ayurvedic medication. The reason for choosing the tincture is its prevalence in being used by several homeopathic doctors. TLC study revealed best combination for better compound separation to be 1:1 n hexane and methyl acetate. A total of 5 compounds were separated among which 2 showed dark bands and were identified as Fraxin and Fraxetin based on their RF values. Therapeutic effect of the sample includes anti cancerous, antioxidant, anti inflammatory and free radical scavenging activity. The results proved the therapeutic potential of the *Fraxinus americana* mother tincture.

All the results were compared and finalized to conclude a good biological role of mother tincture in treating uterine fibroids. MTT assay was performed to detect the efficacy of the sample on viability of uterine leiomyoma cells. The results of this assay revealed the sample showing a measurable decrease in the leiomyoma cell viability conferring its anticancer nature. All the observations made in the study support the use of *Fraxinus americana* mother tincture in the control and treatment of uterine leiomyoma/ uterine fibroids. Thus, the study supports the use of plant based medication, particularly the mother tincture of *Fraxinus americana* in treating cancers and tumors as a better option than surgical removal of fibroids.

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