Chapter 5

DISCUSSION

5. DISCUSSION

5.1 SURVEY OF ETHNOMEDICINE IN WEST SIKKIM

In ethnobotany, the major contribution has been achieved in the field of medicine. A huge amount of knowledge regarding the medicinal plants has remained endemic to certain regions due to the lack of awareness as well as communication (Das *et al*., 2012). Documentation of traditional knowledge which are undisclosed can be useful in understanding the biodiversity (Ramakrishnappa, 2002) as well as making policies for conservation of valuable medicinal plants (Singh, 1999) and this can also be helpful for drug development and natural product research.

In present study, we have compiled the knowledge of medicinal plants used in traditional system by the herbal practitioners of West district of Sikkim due to richness in floral and ethnic diversity of this region. Some villages that came under the four subdivisions of West Sikkim were surveyed. There were many herbal practitioners but 14 of them agreed to share their knowledge for documentation after convincing them. They believe that sharing their herbal knowledge to people other than their family members would make the medicines ineffective. Similar difficulty was faced by Khan *et al*., (2015) when they had conducted survey among the traditional herbal practitioners in Garo Hills, Bangladesh. They have also mentioned that the people of local community were not willing to disclose the name of the plants used to prepare the formulations used by them and they only believe to transfer their knowledge to their next generations.

It was also observed during the survey that in each village some herbal practitioners were available and the people from the surrounding area visited them for treatment of some common illnesses despite having primary health centers in their areas. It might be due to the belief of the people who have probably experienced positive effect of those herbal medicines (Ameade, 2015). Among 14 practitioners interviewed, there was a majority of male noted down as compared to female practitioners who were only 28.5 % in that particular region. This might be due to the fact that females of the house are mostly busy with household responsibilities and raising their children while the male go to search for medicinal plants which are mostly

found in forest or jungle areas. Severe ailments were dealt by the male practitioners while the female members were mostly found to be familiar to medicinal plants or herbal formulations related with common illnesses which they told that they had learnt so as to cure their family members mostly their children when they fell sick with minor health issue.

Most of the practitioners were found to be above the age of 60 years which indicates that the younger generation is not keen on learning the tradition of herbal healing. This might be due to less earning or sometimes no earning from this profession and the younger generation are more interested in higher studies and look for other better source of income (Kala, 2005). Another reason could be the dominance of modern medicinal practice as the educated mass prefers to be treated in hospitals or healthcare centers rather than traditional therapy (Namsa *et al*., 2011). The traditional herbal practitioners are mostly farmers and through our survey also it was found that most of them were dependent on agriculture for their source of income (Kala, 2005) and most of them fell under illiterate category as well. The herbal practitioners provide their services free of cost and the people getting treated by them offer their contribution in the form of vegetables, cereals or some other basic essentials which could be used by them and even if they take the fee that would be in a very less amount as they believe that taking high charges for treatment would deprive the poor from the treatment. Thus the low income from this profession has discouraged the younger generation to carry forward this tradition from their forefathers (Kala, 2005).

From the survey, the total of 36 medicinal plants were documented which were used to prepare 46 herbal formulations. Some of the formulations were prepared from one plant while some comprised of more than one. Altogether there were 9 polyherbal formulations and rest was mono-herbal medicines. Same plant was used in different ailments as well as one plant was used to prepare more than one formulations. The therapeutic activity of plants is credited to the active phyto-components present in the plant parts (Phondani, 2011). In this study, majority of herbal formulations were prepared from root (48 %) followed by other plant parts such as shoot, whole plant, leaf, flower, bark and seed. Roots being used as a main plant part in traditional medicine of

West Sikkim supports the fact that roots are the reservoirs of water and mineral uptake and thus is rich with a number of secondary metabolites such as alkaloids, terpenes, steroids and volatile organic compounds (Rasmann *et al*., 2000). Use of roots has an advantage that it can be available throughout the year regardless of seasonal changes (Mahwasane *et al*., 2013).

Each herbal formulation has to be prepared in a particular method depending on the nature of illness. In this study, most of the herbal formulations were prepared by crushing the fresh plant and obtaining the juice from them (29.73 %) followed closely by dry powder (27.02 %) and remaining formulations were used in fresh form, paste, decoction and infusion. Use of fresh juice could be useful during an emergency such as cuts, rashes, sore throat etc which are very common but also not severe enough to visit a healthcare centre. Some other researchers have also reported frequent use of fresh plants during their survey which could be useful for plants containing volatile oils which are likely to deteriorate if dried or heated (Wilson and Demmig-Adams, 2007; Shetty *et al*., 2006; Namsa *et al*., 2011). Use of dry powder is also quite common as it is a convenient method for storing those medicinal plants which are not available in the surrounding areas. Those plants can be collected in one field visit and stored in powder form so that it can be available throughout the year regardless of change in seasons.

Overall herbal formulations collected from the field were categorized according to the ailments they were associated with. The result revealed that the most prevalent medicines used were related to stomach, indigestion and gastritis which was followed by tonsillitis or sore throat, arthritis and bone related problems, food poisoning etc. There was immense knowledge of herbal medicines which could cure minor to major illnesses. This rich cultural and traditional heritage must be preserved for the future generations to come and it will also enrich the database for researchers who are working on the discovery of new drugs from natural source.

5.2 PHARMACOGNOSTIC STUDY

Herbal medicine has gained a major interest in the recent days as it is considered as a green medicine due to its safety and it is nowadays preferred over the synthetic

drugs which apparently come with harmful side effects. According to recent surveys, almost 50% of the prescribed drugs have originated from natural sources and raw materials (Ranjith, 2018). However there are both merits and demerits of natural drugs. The advantage of naturally obtained medicines is that they are easily available, safe and economic as compared to modern synthetic drugs. The disadvantage of natural drugs is that their growing demand leads to poor quality and adulteration. It might happen due to random collection of the medicinal plants or intentional adulteration with foreign matter to gain more profit. In both ways, it is harmful and may also lead to major health problems while some may not be effective due to collection of wrong plants. Therefore standardization of each herbal formulation is essential in order to check the quality of drugs and avoid adulteration with harmful substances. Standardization can be done using different techniques and methodology such as pharmacognostic study and phytochemical analysis which includes various parameters (Akbar *et al*., 2014). These processes can be helpful in standardization and authentication of the plants or herbal formulations. Authentication for quality assurance of any raw material is necessary to ensure reproducible quality of any herbal materials to provide or justify its efficacy and safety (Ahmad *et al*., 2006; Willow, 2011; Benzie and Wachtel-Galor, 2011; Odugvumi, 2008).

5.2.1 Organoleptic tests

Organoleptic test is the most basic and easy method for the identification of any powder drugs as it is done by the help of sense organs. Since it evaluates the shape, colour, texture, odour and taste of any powder drugs, it can be considered as the quickest and simplest method to ensure the quality and purity of any drugs (Chanda, 2014). In this study we were able to establish a standard for the herbal formulations that we have studied through organoleptic tests so that it can be used as a reference for the authentication of these formulations if it is used in future. Each of the formulation had specific characteristic features which are noted down in table 5.2.1. All of them were odourless but they can be identified through their colour, texture and taste each of which varied from one formulation to the other.

5.2.2 Powder microscopy

Various modern tools are available for the evaluation of plant drugs, but powder microscopy is still considered as the cheapest and simplest method for identification of the plant materials used to prepare the herbal drugs (Kumar and Mannem., 2012). Various plant tissues are observed under microscope after staining powder drugs with particular stains. In this study, there were 11 herbal formulations which were evaluated and all the images of the plant tissues are illustrated in figure 5.2.2.a to figure 5.2.2.k. Some of the prominent features were noted down such as abundant starch granules in TS after staining with iodine solution, cluster of calcium oxalate crystal in stellate shape in AR, plenty of scalariform tracheids in FP, a trichome was also observed under microscope in CB. From these characteristics, it will be easy to compare and authenticate the same formulations as future reference. Powder microscopy is highly recommended parameter for pharmacognostic study because Ayurvedic industries mostly prefer to purchase plants materials or medicinal herbs in dried powder form. Thus it is extremely essential to check the botanical identity of the powdered herbs at first stage as there are higher chances of adulteration in the powder form of herbs. Powder microscopy is economic as well as effortless method to check the quality of drugs at a preliminary stage. This method does not require sophisticated equipment or any expertise required for sectioning and the results are also obtained instantly (Singh *et al*., 2020).

5.2.3 Physicochemical analysis

Physicochemical study is one of the major parameters for the quality control of powder drugs. It evaluates the ash values of the herbal drug along with pH, moisture content and extractive values in water and alcohol. Determination of total ash values indicates the presence of impurities in the formulation due to adulteration or careless processing during the time of drying or storage (Subba and Mandal, 2015). Determination of water soluble ash indicates the presence of inorganic matters, sugars and acids in the powder drugs. On the other hand, acid insoluble ash shows the presence of sand, silica and contamination of the powder drugs with earthy materials which

might have caused due to the inappropriate processing during collection and drying (Ansari, 2006). In this study total ash values of herbal formulations did not exceed 29% but still more improvement can be done and proper care should be taken during the processing time. For water soluble ash, maximum range was at around 23% which shows the presence of inorganic matters present in the plant materials. Acid insoluble ash percentage should be as less as possible and all the formulations had less than 10% acid insoluble ash except for AR and HP indicating the presence of impurities in the form of soil, silica etc which is not acceptable in any herbal drugs that shall be consumed orally. Both these formulations are polyherbal with a mixture of two or more plants and compared to single plant, polyherbal formulation faces more difficulty for standardization (Bhatt *et al*., 2017).

Extractive values were measured in both alcohol (ethanol) and water. These values are helpful in evaluating the chemical constituents present in any herbal drug and to find out which solvent is better to obtain maximum amount of these chemical constituents from the plant materials. Water and alcohol soluble extractive values can be used as standardization parameter as the less extractive values indicates the presence of adulterants (Nisha *et al*., 2017). The extractive value of AA was quite low (2.93 %) which gives a hint of addition of exhausted materials in the formulation. In addition to this, the extractive value also determined that water is a better solvent than alcohol to gain more amounts of extractive weight or phytochemicals from all the studied herbal formulations.

The moisture or loss on drying percentage was also measured to determine water content in the herbal formulations. For higher stability of any herbal formulation, it is essential to have low moisture content. Standard moisture content percentage for any crude drug should not exceed 14% (African Pharmacopoeia, 1986). High content of moisture in herbal formulations encourages the growth of microorganisms such as fungi making it unsuitable for consumption. Moisture content in all the studied herbal formulations were less than 10% which made them suitable for oral consumption. Some herbal drugs can be acidic in nature which is not preferable for consumption and pH of each formulation was measured to ensure the safety of the herbal drugs. As from the

results pH of the formulations did not drop below 4, it was not harmful thus it can be approved for oral consumption.

5.2.4 Fluorescence analysis

Fluorescence is an important phenomenon shown by the phytochemicals present in plants (Gupta and De, 2012). This analysis is based on this behavior of plant constituents which exhibit various fluorescent colours in visible daylight. If they do not attain fluorescence in visible light, they can be illuminated under ultraviolet light because ultraviolet light can produce fluorescence in many natural products and the fluorescent colour is specific to each plant compound. Moreover, some phytoconstituents can be converted into fluorescent derivatives only after they are treated with various chemical reagents (Janchen and Issac, 1988). Since chemicals are not fluorescent, hence we can use this fluorescence behavior of plant compounds to assess the crude drugs qualitatively for pharmacognostic evaluation (Alagar*et al*., 2014; Gupta, 2006; Ansari, 2006). Each of the formulations had specific colours which were noted down along with the images so that it can be used as a reference to authenticate these formulations if ever used in future.

5.2.5 Thin layer chromatography

Thin layer chromatography (TLC) of the herbal formulations was done to identify the active phytoconstituents present in them. The therapeutic activity of any drug is attributed to the presence of secondary metabolites in plants. It is essential to separate and identify those bioactive phytochemicals with any appropriate method. TLC technique is a simple, economical, quick and reproducible method for a preliminary biological and chemical screening of plant extracts. It also provides a fundamental idea about the polarity of the identified chemical constituents (Dutta, 2013).The separation of phytoconstituents is based on their polarity and migration on the mobile phase (Stahl, 2013). The herbal formulations were separated in TLC plates and developed with appropriate reagents after which the presence of various phytoconstituents such as arbutin, anthraglycosides, flavonoids, coumarins, alkaloids etc was observed. The bands were formed on the TLC plates in either visible light or under UV- light (according to

standard methods). These bands were quantified as retention factor (R_f) which is a general characteristic value and it depends or can be changed according to the mobile phase or solvents used for separation (Stoddard *et al*., 2007). The pattern of bands on TLC plates can be used as a fundamental data to display the stability and consistency of herbal components. It is an effective and rapid technique to distinguish the phytochemical classes that may not be fulfilled by other pharmacognostic parameters such as microscopic and macroscopic analysis (Folashade*et al*., 2012). Hence, TLC is the most suggested technique for authentication by creating the fingerprints of herbal medicines due to its specific sensitivity, simplicity, versatility and effortless sample preparation (Mohammad *et al*., 2010)It is also regarded as one of the most convenient method to determine the quality of any herbal drugs and possibly also to identify adulteration in it. The R_f values are specific in a particular mobile phase which could be used as a tool for standardization.

In this study various samples showed the presence of different bioactive phytoconstituents which we have already discussed in results. All the herbal formulation extracts showed the presence of anthraglycosides. It is mainly known for the laxative property in plants thus it can also be useful for the discovery of alternative source of laxative drugs. Researchers have been working for the better source of anthraglycosides in plants. Six anthraglycosides were isolated from Rhubarb (a popular Chinese medicinal herb) made from the root of *Rheum palmatum* L., *R. officinale* Baill. and *R. tanguticum* Maxim (Pharmacopeia, China, 2012; Wang *et al*., 2013). All of these anthraglycosides have revealed to have potential antioxidant activity along with major protective effects during cerebral ischemic injury (Li *et al*., 2004). Similarly, high content of anthraglycosides was observed in the fresh leaves and decoction leaf extracts of *Cassia fistula* and the authors have discussed that it could be useful for exploring a good alternative laxative drugs from natural sources (Sakulpanich and Gritsanapan, 2009).

In some herbal formulations (HP, AR, BP, FP and FF), arbutin was clearly observed as prominent blue bands under visible light. Arbutin is a naturally occurring phenolic glycoside found in many plant species of various families such as Asteraceae

(*Achillea millefolium*), Ericaceae (*Vaccinium* spp., *Arctostaphylos* spp.),Rosaceae (*Pyrus communis* L.) etc. Arbutin is commonly used for urinary disorders and it is very popular as a skin whitening agent (Tomita *et al*., 1990).

Bitter principles are the compounds found in plants which possess a characteristic bitter taste (Evans, 2005). They stimulate all digestive secretions such as saliva, bile, acids, hormones, enzymes, and so forth. Numerous works has been done on therapeutic property of bitter principles. Some of the most commonly known bitter principles are quinine (antimalarial activity), chiratin (antihypertensive activity), calumbin (diarrhea, intestinal gas), boldine (liver disease) etc. (Sharma and Bachwani, 2013). Bitter principles were detected in all the formulation we have studied except three, AA, HS and CB.

Similar to bitter principles, flavonoids were also present in all the formulations except AA, HS and CB. Flavonoids represent as the most widely and commonly distributed phenolic group present in plants (Harborne, 1988). It is the largest group of phenolics compounds occurring naturally in plant parts either in a free state or as glycosides (John *et al*., 2013). These are beneficial for human health system due to their various medicinal properties such as antimicrobial, antioxidant, antiulcer, anticancer activity etc. (Rao *et al*., 2016)

Cardiac glycoside was also detected in the herbal formulations except AA, CB and HS under visible light with blue spots on the TLC plates. Cardiac glycosides occur naturally in plants and venom of a toad species (Steyn and Heerden, 1998) which are steroids that has a powerful action on cardiac muscles which could be helpful for producing beneficial simulation in heart disease thus treating congestive heart failure (Morsy, 2017). Cardiac glycosides have extracted from plant parts have been reported as an anticancer agent by various studies (Kometiani*et al*., 2005; Zhang *et al*., 2008; Prassas *et al*., 2011; De *et al*., 2016). Digitoxin is one of the most popular cardiac glycosides extracted from plants (*Digitalis purpurea*).

Alkaloids were also observed in herbal formulations except AA and CB but the most prominent and maximum number of alkaloid bands was observed in TS. Alkaloids

are naturally occurring group of compounds found mainly in flowering plants containing basic nitrogen atoms and some of which also possess a neutral and a weakly acidic properties (Girdhar *et al*., 2015). Many therapeutic effects of alkaloids have been recorded in previous studies. Many plant derived alkaloids are popular for antispasmodic, analgesic, bactericidal effects (Stary, 1996). It also affects human nervous system with their involvement in the action of chemical transmitters such as dopamine, serotonin, acetylcholine etc (Roberts *et al*., 1998). Some alkaloids such as berberine, sanguinarine have antibiotic activity due to which these are used as antiseptics (Manosalva *et al*., 2014; Croaker *et al*., 2016). Kaur *et al*., 2017 has listed all the plant derived alkaloids and their pharmacological activities such as anticancer, anti-inflammatory, anti-arthritic, anti-diabetic, antioxidant and anti-malarial action.

Coumarins were also detected but only in TS and FF. Coumarins are a large group of plant compounds which are mainly found in higher plants with a major role as natural products, medicinal chemistry and organic chemistry (Monga *et al*., 2012). It is mainly used in pharmaceutical industry as a precursor for the synthesis of anticoagulants (Barcellona *et al*., 2008). Many drugs derived from coumarins compounds have been studied with potential pharmacological activity to treat various types of ailments and these are preferred due to their efficacy, availability, low toxicity, broad spectrum etc (Wang *et al*., 2009). Researchers have been working on the development of coumarins based anticoagulant, antimicrobial (Matos *et al*., 2012; Matos *et al*., 2013), antioxidant (Bubols *et al*., 2013), anticancer (Kapoor, 2013; Vazquez-Rodriguez *et al*., 2013; Xia *et al*., 2013), antidiabetic and anti-inflammatory agents (Bansal, 2013; Matos *et al*., 2013).

Similar TLC technique was used as preliminary step for identifying the presence of phytoconstituents in various studies such as in liverworts (Mukhia *et al*., 2017), some species (aromatic plants) of Lauraceace family (Mandal *et al*., 2016), some herbal formulations used by Rajbanshi community of West Bengal to treat dysmenorrheal pain (Roy *et al*., 2018).

5.3 PHYTOCHEMICAL CONTENT

Phytochemicals are chemical compounds which are non-nutritive and occur naturally in plants during metabolic processes. They have diverse therapeutic properties against various diseases (Minakshi *et al*., 2016). Due to its potential bioactive properties, it has gained a huge attention by pharmaceutical industries. Before exploring bioactivity of any plants, preliminary phytochemical screening is essential to identify new sources of therapeutically as well as industrially important compounds such steroids, alkaloids, phenols, tannins, saponins, terpenoids etc (Akindele and Adeyemi, 2007). In this study, we have determined the presence of phytochemicals in the eleven herbal formulations both qualitatively and quantitatively. Instead of using a single solvent for the extraction of these phytochemicals from plant materials, we have performed successive extraction procedure in ten different solvents which we have discussed in details in 'Methodology' and 'Results' section earlier. Extraction is a crucial step for the extraction of bioactive compounds from plant materials and many studies have supported the influence of variation in extraction processes and solvents used during extraction (Grigonisa *et al*., 2005; Michiels *et al*., 2012). Due to the diverse chemical structure, solubility and polarity of the phytoconstituents, one solvent cannot be considered as standard solvent for extraction and a wide range of extraction solvents with varying polarity is preferred over single solvent extraction (Al-Farsi and Lee, 2008). Quality and quantity of crude extracts, phytochemical content and their biological activities are highly dependent on the polarity of extraction solvent (Do *et al*., 2014; Rafińska *et al*., 2019).

In this study, phytochemicals such as phytosterol, tannin, terpenoids, amino acids, resin, glycosides, cardiac glycosides, reducing sugars, flavonoids and alkaloids were estimated qualitatively. It was observed that most of the phytochemicals were present in moderate to high polar solvents like acetone, butanol, methanol and among non-polar solvents, ethyl acetate and benzene were found to be productive. Phytosterol was detected in herbal formulations; AR, BP, AS, TS and GS with highest quantity in acetone and ethyl acetate extracts. Biological activities of phytosterols have attracted a huge attention in recent times. They have the capacity to inhibit the absorption of LDL

cholesterol from the small intestine and reduce its level in plasma thus lowering the risk of atherosclerosis and protect the body against cardiovascular disease (Calpe-Berdiel *et al*., 2009). There are numerous studies on the dietary phytosterols due to its protective activity against one of the most common but lethal diseases like cancer. Study shows that it decreases the risk of colon and prostate cancer (Jones and Abu-Mweis, 2009) as well as it has immune-modulatory and anti-inflammatory properties (Bouic, 2002). Tannin was detected in BP, AS, TS, GS and FF which was present in high concentration in ethyl acetate, acetone and aqueous extracts. In a similar study, it was found that among polar organic solvents, acetone extract showed highest tannin content in three species of *Acacia* (Elgailani and Ishak, 2016). Tannins can be therapeutically used as anti-diarrheal, anti-hemorrhoidal, homeostatic and anti-inflammatory compounds. In addition to healing burns and stop bleeding of wounds, tannins also help in preventing infection and healing of wound internally (Cheng, 2002). Amino acids were also tested and only few formulations showed its presence which was in limited quantity and it was also seen that mainly water extract and in some cases, methanol and ethanol extract showed the presence of amino acids. This might be due to the better solubility of amino acids in polar solvents. In a previous study, it was established that water is the model solvent for the solubility of amino acids followed by methanol and ethanol (Ji and Feng, 2008). Alkaloids were not detected in any of the formulation except few such as TS. Formulations, FF and AS were also found to contain alkaloids but in a very less quantity and it was extracted mostly in non-polar to medium polar solvents (hexane, benzene, ethyl acetate, acetone), except for FF in which alkaloid was detected in methanol and aqueous extract. We can justify the variation in solvents for extraction of alkaloids on the basis of the fact that there is a variation in polarity of each monomer of total alkaloids thus solvents with different polarity index has to be used to extract total alkaloids. Alkaline alkaloids might have dissolved in water or acidic water and lipophilic alkaloids might have dissolved in non-polar solvents (Yubin *et al*., 2014). Reducing sugars were detected in all the formulations and cardiac glycosides and flavonoids were also detected in all the formulations except for AA, CB and HS. We have already discussed the therapeutic importance of different groups of flavonoids and we have observed in our study that herbal formulations, AA, CB and HS were proved to

be very poor in terms of the presence of different phytochemicals evidenced through TLC technique or qualitative phytochemical estimation. Thus, these three formulations were not taken for further quantitative studies. Another observation in these tests was that the aqueous extract of FF was very consistent for the phytochemical estimation and almost all the higher quantity of phytochemicals were detected in its aqueous extract. As a whole some important phytochemicals such as flavonoids, tannins and glycosides were present in higher concentration in acetone, water and in some cases ethyl acetate. Similar result was observed in the leaf extracts of *Terminalia bellerica* and *Phyllanthus emblica* where acetone, ethyl acetate, water and methanol extracts showed positive results for the presence of the above phytochemicals (Patel *et al*., 2013).

Qualitative tests were done to detect the secondary metabolites present in the herbal formulations but to measure the quantity of these plant compounds, it is essential to proceed further for the determination of other biological properties so that we can sort out those samples which show low concentration of these phytochemicals. Quantitative phytochemical estimation was done on the ten different solvent extracts of eight herbal formulations for the determination of total phenol (TPC), total flavonoid (TFC), total orthodihydric phenol (TOPC), total tannin (TTC), total alkaloids (TAC) and total steroids (TSC) content. Amongst all the formulations, aqueous extract of FF (*F. floribunda*) revealed highest phytochemicals content (TPC, TFC, TTC and TSC). This result is supported by the results of qualitative tests where aqueous extract of FF was found to be the best among other solvent extracts. TOPC was highest in heptane extract of HP while in case of alkaloid content, TS showed highest level of TAC in its ethyl acetate extract. In TLC and qualitative tests also, it was clearly visible that TS has a high quantity of alkaloids and as discussed earlier, it might contain the alkaloids which were lipophilic due to which highest TAC was obtained through ethyl acetate extract. Similarly, in other formulations also which showed alkaloid content, mainly non-polar solvents showed positive impact which again supports the fact that there were higher total amount of lipophilic alkaloids in the studied herbal formulations. On contrary, FF was the only one where TAC was obtained in higher quantity in aqueous extract which indicates the presence of water soluble alkaloids in it.

5.4 ANTIMICROBIAL ACTIVITY

Traditional herbal medicines have been found to have antimicrobial activity and have been dealing with infectious diseases over the years which have led to the search of natural sources of antimicrobial agents and isolate the compounds responsible for antimicrobial activity to replace the synthetic ones. Many phytochemicals derived from plant parts are useful for the development of less toxic and more efficient medicines to control the growth of pathogenic microorganisms including bacteria, fungi and viruses (Kelmanson *et al*., 2000; Ahmad and Beg, 2001). Numerous studies have been done on the antimicrobial activity of various plants extracts and a fair amount of new antimicrobial agents were also discovered (Guleria and Kumar, 2006; Zakaria *et al*., 2007).

Recently the multiple drug resistant strains of microorganism have become a serious trouble in medical field which have been created due to the overuse of antibiotics (Harbottle *et al*., 2006). To reduce the threats of antibiotic resistance microorganisms, discovery of natural antibiotic resistance inhibitors derived particularly from plants is necessary (Kim *et al*., 1995; Alagesaboopathi, 2011). Plants are known to protect themselves from various pathogens by producing various secondary metabolites. In this study, antimicrobial activity of herbal formulation extracts (ethanol and water) were conducted against two gram negative bacterial stains (*Escherichia coli* and *Salmonella typhi*) and three gram positive bacterial strains (*Bacillus megaterium*, *Bacillus subtilis* and *Staphylococcus aureus*). Streptomycin was used as a standard antibiotic to compare the zone of inhibition by the extracts of herbal formulations against the mentioned bacterial strains. The antimicrobial activity was done through disc diffusion method which is the basic and simple method. The results showed the inhibition of *E. coli* by both extracts of GS and FP and the highest zone of inhibition was exhibited by ethanol extract of AR though aqueous extract of AR did not show any inhibition for *E. coli*. Pathogenic *E. coli* is responsible for various diseases such as diarrhea, meningitis, urinary tract infections and sepsis which if not treated can even lead to death (Nataro and Kaper, 1998; Gyles, 2007). *S. typhi* was inhibited only by FP extracts only. Overall study revealed that the extracts of FP and GS were the most

potent antimicrobial agents and especially FP which showed inhibition of all the studied bacterial strains while GS also inhibited all the bacterial strains except *S. typhi*. Interestingly, these two formulations were traditionally used for the treatment of food poisoning (FP) and gastritis (GS). Both *E. coli* and *S. typhi* are the bacterial pathogens which are responsible for major food poisoning and gastroenteritis infecting millions of people worldwide each year (Grassl *et al*., 2008). *S. typhi* causes the infection in intestinal tract and further infecting vital organs such as spleen and liver (Coburn *et al*., 2007). Bacteria of *Bacillus* species are also associated with food borne diseases causing diarrhea, vomiting, nausea, stomach infection etc (Logan, 2011). *S. aureus* is the most common reason for infective endocarditis and also cause skin and soft tissue, pleuropulmonary, osteoarticular infections (Tong *et al*., 2015). The result of antimicrobial activity supports the use of FP and GS in traditional system of medicine for the treatment of food poisoning and gastritis.

5.5 *IN VITRO* CYTOTOXIC ACTIVITY

When a new drug is investigated either from natural sources or synthetic, it should be examined to confirm the safety to the host cell which is known as cytotoxic effect in cancer cell and it is known as cell viability test (Bahuguna *et al*., 2017). Cancer is considered as one of the most alarming disease in recent times being second leading cause of death worldwide (Nataru *et al*., 2014). It is identified by uncontrollable cell growth (Krishnamurthi , 2007) and due to the lack of comprehensive detection methods in early stage, poor prognosis of the cancer patients in late stages and its rapid growth in recent times, it is considered as one of the major health threats or a challenge to mankind in the present scenario (Divisi *et al*., 2006). Treatment of cancer involves surgery, hormonal therapy, chemotherapy, radiation therapy which comes with numerous hazardous health side effects such as hair loss, bone marrow depression, weak immunity, vomiting, headache, anaemia, infertility etc. Natural sources of cancer treatment drugs from traditional herbal medicine are an economical and time saving process instead of screening random plant species (Tan *et al*., 2006). About 50 % of anticancer drugs derived from natural products are already in clinical trials (Cragg and

Newman, 2000). Some popular anticancer agents obtained from plant sources are taxol, podophyllotoxin, camptothecin and vincristine (Pezzuto, 1997).

There are various methods to determine the cell viability of any drugs but MTT assay is the most frequently used method which is based determining cell viability using colorimeter (Mosmann, 1983). Thus we have evaluated the anticancer activity of the bioactive extracts of the eight studied herbal formulations with MTT assay on human liver cell line (WRL-68). MTT assay is the most commonly used *in vitro* assay for the estimation of anticancer activity from plant extracts. This assay is based on the decrease of yellow coloration i.e. MTT and tetrazolium dyes depending on the cellular metabolic activity of NAD(P)H-dependent cellular oxido-reductase enzymes (Berridge, 2005). The healthy cells show a rapid reduction of MTT into formazan while dead or inactive cells have slow rate or no reduction. The end product of MTT reduction gives a purple colour formazan which is dissolved in DMSO and the absorbance of this colour is associated with enzyme activity and to the number of viable cells. High absorbance indicates higher cell viability while less intensity of colour signifies the reduced cell number showing cytotoxic activity of the drug (Berridge, 2005).

In this study, highest cytotoxicity was exhibited by extract of AR amongst all the other herbal formulations with lowest IC_{50} value (173.44±9.82 µg/ml). Studies have indicated that apoptosis, a serious molecular target for prevention of cancer which could be achieved by dietary bioactive agents (Thakkar *et al*., 2014). Since we have already discussed the potential anticancer activity of various phytochemicals present in plants, we may say that the phytochemicals that have been observed and quantified in the phytochemicals determination above could be credited for the cytotoxic activity of these extracts. The cytotoxic activity of plant extracts having < 100 µg ml^{-1} IC_{50} value is usually considered to be therapeutically effective (Hendra *et al*., 2011). Since the lowest IC_{50}value exhibited the extract of herbal formulations against WRL-68 (liver cell line) was 173.44 µg/ml, these herbal formulations would be categorized as weakly active. All the formulations may have exhibited cytotoxic activity but it was not strong enough to be considered as potential anti-cancer agents.

5.6 *IN VITRO* ANTIDIABETIC ACTIVITY

Diabetes mellitus (type-I and II) is a chronic metabolic disorder characterized by the abnormal increase in the concentration of blood glucose level in postprandial and fasting state also known as postprandial hyperglycemia (PPHG) (Klein *et al*., 2007). PPHG is increased by the action of two enzymes, α-amylase and α-glucosidase. Alpha glucosidase is an enzyme present in small intestine which catalyzes the conversion of starch and disaccharides into monosaccharides (Manohar *et al*., 2002). Inhibitors of these enzymes can slow down the carbohydrate breakdown which can be beneficial for diabetic patients (Kwon, 2007). Diabetes is a major health concern worldwide in the present scenario (Gershell, 2005). One of the effective strategies to cure diabetes is by finding α-glucosidase inhibitors from natural products to avoid adverse effects of synthetic drugs that exist in the market (Mccue, 2005). Numerous α-amylase and α-glucosidase inhibitors have already been isolated from plant extracts (Matsui *et al*., 2006; Matsuda *et al*., 2002).

In vitro antidiabetic activity of ten different solvent extracts of eight herbal formulations was evaluated with α-glucosidase inhibiting activity of these extracts. Lower IC_{50} values indicated higher antidiabetic activity and all the herbal formulations inhibited α-glucosidase depending on the various solvent extracts. Acetone, ethanol and aqueous extracts were the most potential solvents for the extraction bioactive compounds which possess antidiabetic activity. The literature review has showed the α-glucosidase inhibitory activity of various phytochemicals such as alkaloids, flavonoids, phenol, terpenes, tannins etc (Yin *et al.*, 2014). From the estimation of phytochemical and antidiabetic activity, it was not possible to exactly point out which particular compound or group of phytochemical is responsible for antidiabetic activity. Different solvent extracts of each herbal formulation exhibited variation in antidiabetic activity. More study is required to isolate and identify the antidiabetic agents from the herbal extracts. Among all the herbal formulations, aqueous extract FF showed highest antidiabetic activity which could be compared with the phytochemical content where aqueous extract of FF was the most potential extract. Interestingly FF was the herbal formulation which was used as potential antidiabetic agent in the traditional system of

medicine which shows that the traditional system of medicine is not random selection of plants and there is a basis of such belief. Both in traditional system and in this study, aqueous extract was the most beneficial solvent and the advantage of using water is that it does not have risk during consumption. Similar result was illustrated by Kazeem (2013) where aqueous extract of *Morinda lucida* demonstrated high inhibition of α-glucosidase enzyme.

5.7 ANTIOXIDANT OR FREE RADICAL SCAVENGING ACTIVITY

Accumulation of free radicals increases oxidative stress which is related with various pathological disorders and diseases including ageing, diabetes, cancer, cardiovascular diseases and neurodegenerative disorders, inflammation, and others (Jadhav HR, Bhutani. 2002; Gulcin, 2002). The most common free radicals or reactive oxygen species (ROS) are hydroxyl radical, nitric oxide, superoxide and lipid peroxyl (Yildirim *et al*., 2000). These ROS lead to radical chain reaction causing the degradation of biomolecules such as DNA, proteins and lipids leading to pathophysiology (Exarchou *et al*., 2002; Afanasev, 2010). These free radicals are trapped by antioxidants thus reducing the damage caused by oxidative stress to the biological molecules (Bektas *et al*, 2005). Recently there is growing interest for the search of natural antioxidants particularly obtained from various plant parts due to the fact that consumption of natural antioxidants lower the risk of oxidative stress related disorders and diseases without countering harmful side effects resulted from the synthetic antioxidants (Baek *et al*., 2004).

Depending on the existence of various free radicals, a single method cannot be sufficient for the determination of radical scavenging activity and /or antioxidant activity in a sample (Erel, 2004). Hence more than one free radical scavenging activity and antioxidant activity were conducted in the solvent extracts of herbal formulations.

DPPH scavenging capacity of the extracts was performed which is based on the ability of a stable free radical, DPPH to decolorize in presence of antioxidants (Hasan *et al*., 2009). DPPH is the most frequently used method because it is easy, rapid and efficient (Mishra *et al*., 2012). This assay determines the ability of a compound to act as

hydrogen donor or free radical scavenger (Kedare and Singh, 2011). All the extracts showed potential DPPH scavenging activity particularly aqueous extract of FF which exhibited lowest IC_{50} value *i.e*. highest scavenging activity. When an electron is donated by an antioxidant compound, the violet colour of DPPH solution is decolorized. Absorbance of this decolorization is measured to quantify the DPPH scavenging activity of the extracts. It has been determined that the free radical scavenging activity is associated with the antioxidant property of phenolic compounds present in the plants such as polyphenols, flavonoids, tannins , terpenes (Rahman and Moon, 2007). It was observed in phytochemical estimation that aqueous extract of FF was the most potential extract for the extraction of phytochemicals such as phenols, flavonoids and tannins. Thus it might be possible that these phytoconstituents could be responsible for the DPPH scavenging activity. Other herbal formulations were also potential DPPH scavengers and the solvent that exhibited the best DPPH scavenging activity varied for each formulation. This result is supported by Roby *et al*., 2013 who concluded in his work on *Thymus vulgaris* that the antioxidant activity of a plant extract or radical scavenging activity are highly affected by the solvents used for extraction of plant compounds

ABTS is another method for the screening of antioxidant activity which could be applicable for both hydrophilic and lipophilic antioxidants because it can be used in both aqueous and organic solvent systems (Samarth and Krishna, 2007). Similar to DPPH assay, this method is based on the decolorization or reduction of ABTS cation by antioxidants (Re *et al*., 1999). It is necessary to perform both the assays because there is a sensitivity differences for capturing these two free radicals depending on the chemical nature of the compounds present in the extract (Faitanin, 2018). This may be justified by the steric blockage phenomenon which may delay or prevent DPPH assay reaction. In this case, when hydroxyl groups in an extract or a sample are closer to the unpaired nitrogen of DPPH radical, there will be a greater ease of reaction which specify that the DPPH scavenging activity can be affected by the variation in structural characteristic of the antioxidant molecules involved (Alisi*et al.,* 2012).

The results of ABTS cation scavenging activity were almost similar to DPPH assay and all the extracts of studied herbal formulations were potential $ABTS^{+}$ scavengers. In this assay, the highest scavenging activity was exhibited by ethyl acetate extract of GS. Phytochemicals such as alkaloids, phenols, flavonoids and orthodihydric phenols could possibly be responsible for this activity as it was observed that these phytochemicals were obtained in highest quantity in the ethyl acetate extract of GS. Other than GS, FP and FF also showed potential $ABTS^{+}$ scavenging activity in moderately polar (acetone) to highly polar solvent extracts. Polar solvents are mainly used for obtaining polyphenols from plant matrix. Aqueous mixtures containing ethyl acetate, acetone, ethanol and methanol are considered as most suitable extraction solvents of plant extracts (Peschel, 2006).

Superoxide anion is known to be a very harmful ROS to cellular components in biological systems as it act as a precursor of more other reactive species (Halliwell and Gutteridge, 2007). Superoxide is produced either in auto-oxidation reactions by enzymatic systems or by non-enzymatic electron transfers where molecular oxygen are reduced in univalent form. It may also reduce iron complexes like cytochromes (Balamurugan *et al*., 2013). They are mildly reactive species but it can be transformed into more reactive and harmful hydroxyl radical in Fenton and Haber Weiss reactions (Aust *et al*., 1985; Babbs, 1985; Deby and Goutier, 1990). Superoxide is mainly produced excessively during inflammation while healthy cells can balance its production (Sies, 1993). However, the over-production of superoxide radicals can be reduced or balanced by chemical or biochemical defenses, otherwise it may lead to the damage of cells involving inflammation and age related diseases (Aust and Svingen, 1982; Tien *et al*., 1982). Thus the role of superoxide in the production of more reactive molecules and its effect in cells suggests the search for potential superoxide scavengers particularly from natural sources (Murakami *et al*., 2000; Perry *et al*., 2000).

The superoxide scavenging activity of various extracts of herbal formulations were measured by the reduction of nitro blue tetrazolium/NBT (Robak and Gryglewski, 1988). In this study, the most potential superoxide scavengers were obtained through aqueous extract of FF. In fact in majority of the formulations, water was the most

suitable solvent for extraction of superoxide scavengers as aqueous extracts of HP, AR, AS, TS and FF showed highest scavenging activity. Polar solvent have always been considered to be suitable for the extraction of polyphenols from matrices and the extraction of polyphenols from natural products depends highly on their solubility in a particular solvent (Naczk and Shahidi 2006). On the other hand, ethyl acetate extract of GS and FP showed highest superoxide scavenging activity while for BP, benzene extract was the best for this activity. All the phenolic compounds particularly flavonoids are considered as potential superoxide scavengers (Robak and Gryglewski, 1988). Interestingly in these formulations, the same extracts exhibited highest total flavonoids content.

Nitric oxide is another reactive free radical which plays a vital role in inflammatory processes and also involved in regulation of other various physiological processes. It is generated by endothelial cells, phagocytes, neurons (Gangwar *et al*., 2014). Nitric oxide (NO), if produced continuously, causes cell injury and tissue toxicity leading to vascular collapse and when it is produced excessively, it causes various complications associated with different types of carcinomas and inflammatory condition along with arthritis, juvenile diabetes, ulcerative colitis, multiple sclerosis (Taylor *et al*., 1997). Biomolecules such as DNA or proteins may not be directly affected by the production of NO as its toxicity increases only when it reacts with superoxide to produce peroxynitrite anion which is considered to be genotoxic (Wink *et al*., 1991). The overproduction of NO intermediates can be balanced by the antioxidants preferably from natural sources such as plants. In this study only some herbal formulations were capable of scavenging NO radical which also varied according to solvents used for extraction. Among those extracts, aqueous extract of FF again stood out with highest scavenging activity. Flavonoids and phenolic compounds present in plants are known to possess NO scavenging property (Kim *et al*., 1999; Crozier *et al*., 2000; Madson *et al*., 2000; Jagethia *et al*., 2004), thus we can speculate that phytochemicals present in the plant parts of the above herbal formulation might be accountable for the observed NO scavenging activity.

There are certain human diseases that require blood transfusion such as anemia, thalassemia for treatment and survival. Excess iron is released by the breakdown of red blood cells of the transfused blood and deposited as hemosiderin and ferritin in spleen, liver, endocrine organ and myocardium. Human body is unable to eliminate these iron storage complexes and the excess deposition of these iron complexes can cause damage of cells leading to complications such as hypothyroidism, liver failure, diabetes, heart failure(Taher *et al*., 2006; Rund and Rachmilewitz, 2005; Loukopoulos, 2005). When ferrozine reacts with Fe^{2+}, iron complexes are formed which however can be disrupted by the presence of chelating agents. The metal chelating activity of an extract can be quantified from the absorbance taken when the pink colour complex is reduced by the action of chelator (Ebrahimzadeh, 2008). Phyoconstituents are known to have metal chelating potential which could have beneficial impact on metal catalyzed biochemical reactions like protein auto oxidative glycation, fragmentation and glycoxidation reactions (Ghous *et al*., 2015). It was observed in results that not all the herbal formulation we have studied had metal chelating activity. It was influenced by the solvents used for extraction as only few solvent extracts of some herbal formulations exhibited metal chelating activity. Only in case of AS and FF, all the extracts showed chelating activity. The most active extract which interrupted the complex of ferrozine and ferrous ion was aqueous extract of FF indicating that the compounds present in it could capture the ferrous ion before ferrozine.

Ferric reducing antioxidant power (FRAP) is associated with the antioxidant activity and provide a reflection of antioxidant capacity (Oktay *et al*., 2003). FRAP assay measures the reducing power of an antioxidant by converting Fe^{3+}/ ferricyanide complex formed in this assay to the Fe^{2+} form. In this method, yellow colour solution of reaction mixture changes to green or dark blue in colour depending on the reducing potential of the compound. The FRAP of a compound is measured by determining the absorbance of the colour of final product at 700nm in which higher absorbance indicates higher reducing power or antioxidant activity (Vijayalakshmi and Ruckmani, 2016). In this study all the herbal formulations exhibited reducing power but in particular solvent extracts only except HP and FF in which all the solvent extracts showed tremendously high reducing power. In HP, acetone extract showed highest

reducing power and in FF, the aqueous extract exhibited the best. FRAP is an antioxidant determining assay and based on electron transfer, it is also known as redox linked colorimetric method. Thus, compounds possessing reducing power are recognized as electron donors as they can reduce the oxidized intermediates produced from lipid peroxidation processes (Pietta, 2000). The reducing potential of any compound could be due to the presence of polyphenols, flavonoids in plants as many researchers have widely reported the polyphenol structure and its relationship with ferric reducing capacity (Benzie and Strain, 1996; Parejo, 2003). Phenolic compounds are also known as antioxidants because of their capacity to reduce or prevent lipid peroxidation inhibition, scavenge free radicals and reducing effect (Maksimovic *et al*., 2005).

5.8 ANTI-HYPERTENSIVE ACTIVITY

In recent time, hypertension is one of the major health concern worldwide causing mortality associated with coronary artery disease and its complications such as renal failure, heart failure, stroke and diabetes (Abegaz *et al*., 2017). To reduce the risk of hypertension and the health complication related to it, a dietary and lifestyle changes are recommended. However when the lifestyle changes do not improve the condition, drug treatment is essential for patients (Bazzano, 2008). Some of the common and effective antihypertensive drugs are β-blockers, diuretics and calcium antagonists but they are also associated with harmful side effects (Atkinson and Robertson, 1979). The activity of angiotensin-I converting enzyme (ACE) helps in the regulation of blood pressure (Gohlke *et al*., 1994). ACE converts an inactive decapeptide, angiotensin-I into angiotensin-II, bradykinin (hypotensive peptide) to inactive components (Skeggs *et al*., 1956). This reaction is catalyzed by ACE and the inhibitors of ACE can prevent the formation of angiotensin-II by ACE leading to the reduction of vascular resistance and blood pressure. Inhibition of ACE is established as a therapeutic principle for the treatment of hypertension (Hansen *et al*., 1995). In this study, only two herbal formulations (BP and FP) out of eight formulations exhibited antihypertensive activity. Benzene extract of BP showed the highest antihypertensive activity and in case of FF, acetone and water extracts showed the best antihypertensive activity. Interestingly, BP

was traditionally used for the treatment of high blood pressure which justifies its use in tradition system as well as opens a scope for exploring antihypertensive drug for *in vivo* methods followed by purification of the extract to isolate compound responsible for its bioactivity. Many bioactive constituents present in plants have ACE inhibitory activity such as flavonoids, tannins, alkaloids, terpenoids (Somanadhan *et al*., 1999).

5.9 CORRELATION

5.9.1 Pearson correlation

Polyphenols are one of the most abundant compounds found in plants. Phenolic compounds are generally responsible for antioxidant property and other biological activities such as inhibition of certain enzymes involved in various diseases (Russo *et al*., 2015). The secondary metabolites such as tannins, flavonoids, glycosides etc present in plants or plant extracts have been reported to have antidiabetic property (Suba *et al*., 2004). Pearson's correlation coefficient was done to evaluate the relationship between polyphenolic compounds with antioxidant and antidiabetic activities. It was observed that radical scavenging activity such as DPPH showed a negative correlation with the amount of phenolic compounds such as TPC, TTC and reducing power (FRAP) which clearly indicates the effect of these compounds i.e. phenols and tannins in the scavenging activity of free radicals. The negative correlation between the IC_{50} values in radical scavenging assays and antioxidant activity shows that with the increase of phenolic compounds, its antioxidant potential to scavenge free radical will also increase. Several earlier studies have also showed similar correlation between polyphenols and antioxidant activities. Chaudhari and Mahajan (2015) showed correlation between the phenolic compounds present in 20 Indian medicinal plants and its reducing powder and its potential to neutralize DPPH free radical. More such results were shown in literature reviews where there is positive correlation between the amount of polyphenols and antioxidants in plant extracts (Sagar and Singh, 2011; Liu *et al*., 2009). The negative correlation between the IC_{50} values of DPPH free radical scavenging assay and phenol content was also reported by Quiroga *et al*., (2013) during the investigation of the essential oils of *Lippia turbinata* and oregano. Since antioxidants of phenolics have

been known for redox potentiality and thus are considered as hydrogen donors, free radical scavengers and also as redox agents (Macheix JJ, Fleuriet, 1998). The entire mechanism of phenol content evaluation by Folin-Ciocalteu method is based on the reducing properties of these compounds which is also evident in this study where a strong positive correlation was observed between TPC, TFC, TOPC, TTC, FRAP. Similar result was reported by Subba and Mandal (2019) in the study of a traditional herbal formulation where these variables were positively correlated with eachother.

5.9.2 Principal Component Analysis

Principal component analysis (PCA) is a technique which uses multivariable to analyze a data and the results are described by various quantitative dependent variables that are inter-correlated with eachother. It basically transforms the measured variables to new uncorrelated variables known as principal components of similarity and differences between various groups (Cam *et al*., 2009). In this study PCA was plotted to determine the visual similarity among the phytochemical content, antioxidant and antidiabetic activity of the studied herbal formulations. In PC1, free radical scavenging activities like DPPH, SO, reducing power (FRAP) and phytochemicals like TPC, TFC and TTC were heavily loaded indication their inter-relationship with eachother. It was evident from the Pearson correlation also that these phytochemicals could probably be responsible for the free radical scavenging activity of the extracts. Similar result was obtained when PCA was conducted to observe the similarity and differences among 18 different cereals on the basis of four variables such as FRAP, CUPRAC, phenols and flavonoids (Cam *et al*., 2009). In PC2, antidiabetic activity determined with IC50 values was found to be loaded along with NO diverged in two different directions indicating the complexities if diabetes related with NO scavenging activity. Several studies have reported earlier that hyperglycemia may enhance the production of NO (Adela *et al*., 2015; Savino *et al*., 2006; Shahid and Mahboob, 2009). Overall, PCA in this study clearly suggests that the phenolic compounds and free radical scavenging activity are interrelated and determines the antioxidant properties of the these phenolic compounds.

5.10 INFLUENCE OF EXTRACTION METHODS ON THE BIOACTIVITY OF *FRAXINUS FLORIBUNDA*

Numerous active compounds such as alkaloids, tannins, steroids, glycosides, phenols, flavonoids, volatile oils are deposited in specific plant parts such as flowers, root, leaves, bark, fruits, seed etc and the therapeutic as well as other beneficial properties of plants are credited to these compounds and its combination (Tonthubthimthong *et al*., 2001). An appropriate extraction method and solvent system has to be followed to obtain the bioactive phytochemicals with high efficacy and efficiency. Efficacy refers to the bioactivity or potency of the extract while efficiency means the percentage yield of the extract (Jadhav *et al*., 2009). The selection of suitable method for the extraction or isolation of plant components with best yield and high purity from natural products is generally dependent on the nature of compounds or the plant material used (Kothari *et al*., 2009).There are numerous evidences of extraction methods influencing the bioactivity of the plants (Recknagel *et al*., 1989; Weber *et al*., 2003). A strong positive correlation was found between antibacterial activity and extraction efficiency while investigating bioactivity of plant seed extracts (Kothari *et al*., 2010). The same plant samples may exhibit a variation in their bioactivity when processed through different extraction methods. Antioxidants activity of some spices was enhanced in cold percolation method while in the same plants materials, antimicrobial activity was higher in hot water extraction method (Abdelfadel *et al*., 2015).

In this study, bark of *Fraxinus floribunda* (BOFF) was extracted through different methods to access the influence of bioactivity of the extracts. This plant was selected on the basis of the previous results where the aqueous extract of BOFF was the most potential herbal formulation as compared to the other formulations. As we have already discussed that there were four methods used in this study viz., autoclave boiling under pressure (AB), in a soxhlet apparatus (S), boiling at normal pressure (NB) and in cold condition (CP). We have evaluated the bioactivity of these extracts with phytochemical content, antioxidant activity and antidiabetic activity.

5.10.1 Antioxidant activity

Aqueous extract of *F. floribunda* has exhibited potential antioxidant activity and it was observed that the variation in extraction method has clearly influenced the bioactivity of BOFF. Another observation was the retention of bioactivity till the third stage since the same plant material was extracted successively for thrice. The highest antioxidant activity from overall study was shown by the extract obtained through AB (pressure boiling) with highest free radical scavenging activity, reducing power and total phenol content. This result is comparable with the phenol content observed in ginseng seeds which was three times higher when extracted through autoclaving at 130°C than the untreated seeds (Bae *et al*., 2012). The phenolic compounds present in plant materials could have been diffused from the seed coats after applying continuous pressure for a long time leading to the elevation of tocopherols, tocotrienols, vanillin, ferulic acids, p-coumaric acids etc. (Bryngelsson*et al*., 2002). Flavonoid and SO scavenging activity were highest in NB while soxhletion was suitable extraction method for ortho-dihydric phenol content. Only the extract obtained through cold percolation was not impressive enough. The reason behind it could be because of the advantage of high temperature over cold for extraction of bioactive compounds. High temperature enhances the extraction process by the decrease in viscosity of liquid solvent resulting better penetration of matrix particles. High temperature disrupts the solute-matrix interactions caused by hydrogen bonding, Van der Waal forces, dipole attraction and active sites on the matrix (Kuzmanovica *et al*., 2015). However, when pressure is applied on high temperature, it enables the solvent to reach into certain areas of matrices which would not be reached under atmospheric condition (Casazza *et al*., 2012). Another advantage of high-pressure boiling is that it requires less time, energy and it has also been recognized as environment friendly technology by FDA (Food and Drug Administration) and very appropriate for large scale extraction in pharmaceutical, food and metallurgical industry (Heldman and Busta, 2000).

It is known that phytochemicals present in plant parts act as antioxidants, thus we have estimated the phenol, flavonoid and orthodihydric phenol content of all the process variation extracts of BOFF. All these three phytochemicals were obtained

through these extraction methods but highest phenol and flavonoid content was exhibited by pressure boiling. It has indicated the involvement of phenolics for the antioxidant activity above.

Although pressure boiling was found to be suitable for the recovery of antioxidants from BOFF but the retention of bioactivity was observed in extracts obtained through soxhlet as it retained the bioactivity till third stage. However, the pressure boiled extracts showed high bioactivity at first stage which could not be seen in that good amount till third stage. The first stage of pressure boiled extract was visibly darker in colour when compared with other extracts while the third stage extract from pressure boiling was very faint in colour. This might be due to high extraction yield at high pressure at the first stage itself. Extraction yield can be increased on applying high pressure (Dornenburg and Knorr, 1993) as high pressure can deprotonate charged groups and break salt bridges and hydrophobic bonds present in cellular membranes leading to higher permeability (Ahmed & Ramaswamy, 2006). Another observation during extraction was that the pressure boiled extract was the only extract which was clearly visible but all the other extracts were hazy and cloudy. Similar observation was reported in the fruit of *Litchi chinensis* where the pressure boiled extract was cloudy and very dark in comparison to other extracts (Prasad *et al*., 2009).He explained phenomenon on the fact that large molecules such as starch and proteins were denatured under the condition of high pressure and heat together and thus did not move into the solvent. A clear extract solution is highly preferred for experiments based on absorbance of the colour of a reaction mixture.

5.10.2 *In vitro* antidiabetic activity

We have already discussed that aqueous decoction of the bark of *F. floribunda* is popularly used in traditional medicine for treating diabetic patients. Result of successive solvent extraction for *in vitro* antidiabetic activity done above also revealed that this formulation is a potential antidiabetic agent. Before proceeding towards the *in vivo* assays, the most suitable and productive extraction method to obtain compounds from BOFF with highest bioactivity on the basis of antioxidant and antidiabetic activity was

determined. For antioxidant activity, pressure boiled extract was the most productive method. *In vitro* antidiabetic activity was conducted by α-glucosidase enzyme inhibiting activity of the extracts. Similar to antioxidant activity, highest antidiabetic activity was also shown by the pressure boiled extract of BOFF. There are studies and clinical evidences that excessive ROS generation has been observed in diabetes (Type-I and Type-II). This indicates the association of diabetes occurrence with oxidative stress caused mainly through oxidation, oxidative degradation of glycated proteins and non-enzymatic protein glycation (Johansen *et al*., 2005; Rosen *et al*., 2001). Increase of ROS in the body weakens the antioxidant defense mechanism which leads to enzymatic and cellular damage and lipid peroxidation ultimately causing the development of insulin resistance and hyperglycemia (Halliwell, Gutteridge, 1990). Numerous studies have reported the ability of antioxidants to improve insulin action (Paolisso *et al*., 1994; Paolisso *et al*., 1993; Faure *et al*., 1997). Various phytochemicals present in plants are known to possess antidiabetic effect (Malviya *et al*., 2010), particularly flavonoids which are suggested to be beneficial for managing diabetes mellitus (Ceriello, 2000; Nicolle *et al*., 2011). As we have mentioned above that pressure improves the permeability and solubility of plant tissue and also increases the diffusibility and better movement through the cellular components, it is suggested that this extraction method was useful for the extraction of antidiabetic compounds for the extract.

5.11 PHARMACOLOGICAL ASSAYS

Pharmacological assays such as anti-inflammatory, hepatoprotective activity and antidiabetic activity were conducted in animal model. The extract obtained through pressure boiling was selected for these *in vivo* studies based on its performance during *in vitro* assays. Diabetes mellitus is a disease related with several complications associated with multiple organ systems leading to irreversible pathological conditions including liver congestion (hepatopathy) and inflammatory diseases (Reid, 2006; Donath and Shoelson, 2011; Zozulinska and Wierusz-Wysocka, 2006). Numerous studies have reported the association of diabetes mellitus with liver abnormalities such as fibrosis, cirrhosis, non-alcoholic fatty liver, hepatocellular carcinomas, viral hepatitis, abnormal elevated hepatic enzymes (Papatheodoridis *et al*., 2006; Pickup, 2004).

Hyperglycemia and a fatty liver can destroy the hepatocytes and increase the rate of morbidity and mortality among diabetic patients (Levinthal and Tavill, 1999). Inflammatory responses have contributed to the development of diabetes mellitus and some even argue that diabetes mellitus is a manifestation of an existing low-grade inflammation (Donath and Shoelson, 2011; Zozulinska and Wierusz- Wysocka, 2006). There was in fact a statistically significant relation of diabetes mellitus with the decreased occurrence of some common inflammatory diseases such as asthma, chronic hepatitis, chronic gastritis or ulcer, chronic gastroenteritits etc. (Zheng *et al*., 2015).

Since diabetes is associated with complications in liver and inflammatory diseases, natural antioxidants particularly from plants with a potential antidiabetic, hepatoprotective and anti-inflammatory diseases could serve as a solution for many life-threatening health conditions.

5.11.1 Anti-inflammatory activity

Inflammation is condition which is caused due to the reaction of living tissues during injury. In this study, we have used the carrageenan-induced rat paw edema model for anti-inflammatory study which is believed to be a biphasic process. The initial phase of 1-2 hr inflammation is induced by carrageenan due to the synthesis of serotonin, histamine, and prostaglandins in the surrounding of the injured tissues. The second phase is continued by prostaglandin synthesis and mediated by leukotrienes, bradykinin, prostaglandins, and polymorphonuclear cells produced by tissue macrophages (Brito and Antonio, 1998). The leaves and the bark of *F. floribunda* are used for the treatment of gout, bone fracture and dislocation in folk medicine (Gurung, 2002). Anti-nociceptive and anti-inflammatory activity was reported in the leaves of FF (Lingadurai *et al*., 2007) but there was not a single scientific report or study in the bark of this plant despite being popularly used on traditional medicine. The results showed potential anti-inflammatory activity by BOFF in the later stages after 2 hours which indicated that the activity might have been attained due to the inhibition of prostaglandins. The second phase of oedema is reported to be responsive to most clinically effective anti inflammatory drugs that are frequently used to access analgesic

effect of natural products (Loggia *et al*., 1986).Since flavonoids are reported to reduce or prevent prostaglandins synthesis (Middleton *et al*., 2000; Havsteen, 2002), the anti-inflammatory activity of BOFF might be due to the flavonoids present in the sample as reported in quantitative estimation of total flavonoids.

5.11.2 Hepatoprotective activity

Carbon tetrachloride (CCl_4) is a common hepatotoxin which is popularly used to induce liver injury in laboratory animal models to study hepatoprotective effect of plant extracts or drugs (Osadebe *et al*., 2012). When CCl_4 is activated, the free radical reactions are also initiated which causes oxidative stress and lipid peroxidation (Tirmestein *et al*., 2007) which is assumed to be the basis of the development of CCl_4 induced hepatotoxicity (Poly *et al*., 1987). CCl_4 toxicity is caused due to the transformation of free radicals into trichlorimethyl radicals that binds with or attacks polyenoic fatty acid in liver membrane (Slater, 1984; Halliwell and Gutteridge, 1984). Damage or injury in hepatocytes is indicated by the increased level of serum SGOT, ALP, SGPT and bilirubin (Giannini *et al*., 2005). These enzymes are present in liver in high concentration mainly due to the necrosis of hepatocyte or abnormal membrane permeability and are released from cells to the blood (Nkosi *et al*., 2005). SGPT is a very sensitive indicator of severe liver damage and SGPT is more selectively a parenchymal enzyme of liver than SGOT (Shah *et al*., 2002). The elevated level of these enzymes was observed in group I *i.e*. CCl_4 treated rats were caused due to the excessive liver damage by the toxins. Treatment of hepatic rats with the extract of BOFF at a dose level of 100 mg/kg b.w.was able to improve the hepatic damage caused by CCl_4 when compared with standard formulation Liv-52. The level of SGPT, SGOT and bilirubin in hepatic rats after treatment with Liv-52 and BOFF extract were almost equal in quantity which indicated that the extract is a potential hepatoprotective agent. The reduced level of SGPT, SGOT and ALP in extract treated rats in this study might be due to the presence of flavonoids which we were observed in previous studies also. A number of flavonoids were accounted for anti-inflammatory activity *in vitro* and *in vivo*. The important mechanism of flavonoids for anti-inflammatory activity is due to the inhibition of eicosanoid generating enzymes such as cyclooxygenases, lipoxygenases

andphospholipase-A2 which in turn reduces the concentration of leukotrienes and prostanoids (Kim *et al*., 2004). Other mechanisms are inhibition of phosphodiesterase, protein kinases, histamine release and activation of transcriptase (Rathee *et al*., 2005). Bilirubin level is the most useful indicator to check the severity of liver damage and the decreased level of serum bilirubin in extract treated hepatic rats indicated the effectiveness and benefits of BOFF extract for a normal function of liver. Hepatoprotective drugs from plant sources contain a variety of phytoconstituents such as phenol, flavonoids, alkaloids, carotenoids, xanthenes, monoterpenes etc. (Gupta and Misra, 2006).

5.11.3 *In vivo* antidiabetic activity

In vitro antidiabetic assay have already revealed the potential antidiabetic activity of BOFF extract. In present work, we have further investigated the antidiabetic property of BOFF extract in animal model which was performed on the streptozotocin (STZ) induced diabetic rats. The STZ induced diabetes mellitus in rat model is a commonly used and widely accepted method due to its resemblance with diabetes mellitus (Adisa *et al*., 2011).

There was an improvement in glucose tolerance test with the reduction of plasma glucose level which indicates the insulin-mimetic activity or improvement of glucose utilization mechanism by the extract (Singh *et al*., 2018). The antidiabetic activity of FF extract might be credited to the decrease in damage of pancreatic β-cells, thus improving the production of insulin from the β-cells of the pancreas. Numerous plants have been previously reported to have anti-hyperglycemic activity by insulin stimulating effect. (Jia *et al*., 2009; Gireesh *et al*., 2009). The mechanisms of actions for plants with anti-hyperglycemic activity mainly include the increase of insulin secretion, control in glucose absorption by the intestine, more glucose absorption by muscle and fat tissues, and control in the production of glucose from liver cells (Krishnamurthy *et al*., 2011). One of the characteristic features of diabetes is the inability of glucose uptake by the muscle cells because of low insulin production which consequently causes muscle wasting and decrease in bodyweight. Induction of STZ in rats will

destroy the pancreatic β-cells due to low levels of insulin (Baruah *et al*., 2017). Thus, there was a decrease in bodyweight in diabetic control rats as compared to normal control ones, which indicate theexcessive breakdown of tissue proteins causing the loss of body weight in diabetes (Sajeesh *et al*., 2011; Kumar *et al*., 2011; Poongothai *et al*., 2011; Mishra *et al*., 2011). It was clearly observed that the administration of BOFF improved the loss of bodyweight which indicated the control on wastage of muscle in diabetes. The BOFF extract has probably stimulated the pancreatic beta cells leading to the production of insulin. Diabetes affects lipid profile, and the most common lipid abnormalities are high TGL and high TCL. In this study, there was an increase in TCL and decrease in HDL in diabetic control rats. Deficiency of insulin may cause the failure to activate lipoprotein lipase resulting into hypertriglyceridemia (Appalaraju *et al*., 2011). However, the bark extract of FF was able to control the lipid levels in diabetic rats. In diabetes, LDL brings cholesterol to the peripheral tissues to be deposited while HDL carries cholesterol to liver from peripheral tissues and helps its excretion. LDL is responsible for the deposition of fats in arteries. In this study, we have observed a significant decrease in TCL, TGL, and LDL, whereas HDL was significantly increased. The liver is important and helps the body in controlling blood glucose with glycogenesis and glycogenolysis. The liver sections of STZ-induced rats revealed various architectural changes in the liver with inflammation of sinusoids, changes in central veins, and portal area with vacuolization of cytoplasm. Similar findings were reported earlier by many researchers with histopathological changes in the liver (Aboonabi *et al*., 2014; Hassan *et a*l., 2018; Ania *et al*., 2017). Unlike diabetic control, the liver sections of diabetic rats treated with glibenclamide and BOFF showed less histopathological changes and improved liver architecture. It indicates the protective effect of the extracts to control hepatic injury during diabetes.

5.12 BIOASSAY GUIDED PARTIAL PURIFICATION OF BARK OF *FRAXINUS FLORIBUNDA*

Bioassay guided purification by fractionation of plant extracts associated with chromatographic techniques of compound separation can direct the isolation of bioactive molecules. New strategies of fractionation for the discovery of principal

compounds which can be used as a potential source of new drugs are being developed in recent times. Numerous bioactive compounds have been isolated from the crude extracts of different groups of plants through bioassay guided purification. Hajdu *et al*. (2010) isolated three compounds Eudesmanolide sesquiterpene, sivasinolide 6-O-angelate and centaureidin from *Anthemis ruthenic*a M. which has anticancer activity. Similarly methyl protocatechuate, patuletin and patulitrin were isolated from *Tagetes patula* which was observed to havepotential antioxidant with analgesic properties (Faizi *et al*., 2011). Again, an anti-ulcer compound, Tagitinin C was isolated from *Tithonia diversifolia* Hemsl. (Sanchez-Mendoza *et al*., 2011). Among volatile components of *Rhododendron arboretum*; 9, 12-Octadecadienoic acid, methyl ester was isolated which was found to possess hepatoprotective, hypocholesterolemic, antihistaminic and anti-eczemic properties (Painuli *et al*., 2016).

The traditional use of *F. floribunda* bark as an antidiabetic agent gave an idea to frame antioxidant and antidiabetic assay guided purification of bioactive substances from the extract of the same. It is known that plants' extracts are the storehouse of numerous bioactive phytochemicals and each of them are varying with different polarity indices. Thus in this work, solvent with lower polarity were sued first to separate lipids initially followed by higher polarity solvents to elute more polar substances from the extract. Lower to higher polarity solvents was also used by Jassbi *et al.*, 2016 for isolation of bioactive phytochemicals. Kanagavalli and Mohamed Sadiq (2018) have performed similar techniques for isolation of bioactive compounds from *Boerhavia diffusa* Linn. by using different polarity solvent through column chromatography. After conducting various assays, one bioactive fraction was selected on the basis of high pharmacological activity and phytochemical content. When this fraction was passed through Gas Chromatography Mass Spectrometry (GC-MS), some active phytochemicals present in the extract of *F. floribunda* bark was detected.

5.12.1 GC-MS analysis and identification of phytochemicals

GC-MS analysis of the extract of *F. floribunda* bark revealed the presence of various groups of phytochemicals such as hydrazone derivative, terpene, flavonoids,

coumarin along with resin and fatty acids. The potential antioxidant, antidiabetic, anti-inflammatory and hepatoprotective activity of this plant could be attributed to the presence of these phytochemicals. 2(1H)-Quinolinone, hydrazone also known as 2-hydrazinoquinoline is a heterocyclic aromatic organic compound which is a hydrazone derivative was found in *F. floribunda* extract. It was the most abundant compound in the extract. Many hydrazone derivatives have been reported to possess biological activities (Singh and Raghav, 2011; Saleem *et al*., 2011). We have already discussed that NSAIDS are largely used for the treatment of inflammation and pain. However, compared to NSAIDS, hydrazones which are inhibitors of both cyclooxygenase (COX)and 5-lipoxygenase (5-LO)are given more interest and being studied as a potential analgesic and anti-inflammatory agent (Almasirad *et al*., 2005).Various hydrazone derivatives were synthesized by Moldoven *et al*., (2011) which showed potential *in vivo* anti-inflammatory activity. Uno *et al*. (1995) synthesized 2(1H)-quinolinone derivatives and was found to be a potent inhibitors of 12(S)-hydroxyeicosatetraenoic acid (12-HETE) which has key role in the pathogenesis of numerous circulatory disorders and arteriosclerosis. A series of 2(1H)-quinolinone was synthesized by Koga *et al*., (1998) for the evaluation of anti-thrombotic and anti-hyperplastic activities from which 1p (OPC-33509) was best for the anti-arterostenotic agent. Sugarhydrazones of 2-hydrazinoquinoline were evaluated and reported to have antimicrobial activity against some Gram +ve and Gram –ve bacteria such as *Staphylococcus* sp., *Bacillus* sp., *Pseudomonas* sp etc. (Khodair *et al*., 1998). Some hydrazone derivatives were also found to have potential anti-hyperglycemic activity. A set of heterocyclic hydrazones were synthesized to inhibit glycogen synthase kinase-3 (GSK-3) which is a serine/threonine kinase associated with diabetes by inactivating glycogen synthase that converts glucose into glycogen (Smalley *et al*., 2006). GSK-3 inhibitors are regarded as a new approach for the treatment of diabetes.

The extract also revealed flavones in it, which are a class of flavonoid. Flavones were associated with various pharmacological activities such as antioxidant, antidiabetic, antibacterial, and anti-inflammatory activities. Some flavones synthesized by Goker *et al*., (2005) showed potential antimicrobial activity against *E. coli, S. aureus, S. faecalis* etc. In the methanolic extract of *Artocarpus heterophyllus*, two

flavones 6-(3-methyl-1-butenyl)-5,2′,4′-trihydroxy-3-isoprenyl-7-methoxyflavone and 5,7,2′,4′-tetrahydroxy-6-isoprenylflavone were identified which actively inhibited the growth of carcinogenic bacteria and plaque- forming streptococci (Sato *et al.*, 1996).In another study, flavonol like santin was reported to have an inflammatory activity of *Tanacetum parthenium* leaves (Moroney and Somanchi, 1999). The isoflavones such as genistein was found to possess oestrogenic activity and in later research, another flavonone8-isopentenylnaringenin isolated from a crude drug which was derived from *Anaxagorea lutzonensis* wood was discovered to have better oestrogenic activity than genistein (Miyamoto *et al.*, 1998). Many plant extracts containing flavones were reported to have hypoglycemic activity too. Some synthetic analogs of chrysin exhibited antidiabetic activity in diabetic mice (Shin *et al*., 1999). An aryloxypropanolamine derivative of 7-hydroxyflavones was synthesized by Pratap *et al*., 2009 which also showed significant glucose-lowering effect in db/db mice. Flavones have always been considered as potential antioxidants with polyphenolic flavones being superior free radical scavengers. Remarkable antioxidant activity of several flavones such as Wogonin (Chen *et al*., 2004), Apigenin (Oteiza *et al*., 2005), Luteolin (Shahidi *et al*., 1992) have also been reported.

The next compound identified in the extract was Coumarin,6-amino-3-phenyl. Coumarins are large class of secondary metabolites found in higher plants and is considered to have various biological properties such as antibacterial (Canning *et al*., 2013), anti-inflammatory (Witaicenis *et al*., 2014), anti-neoplastic activity (Nasr *et al*., 2014: Bronikowska *et al*., 2014) etc. Coumarins have captured more attention in terms of biological activity for having several positive effects in diseases with less cell damage (Bilgin *et al*., 2011). Coumarins and its few derivatives are well known for various industrial and medical purposes. Coumarin, 6-amino-3-phenyl also known as 2H-1-benzopyran-2-one is an oxygen heterocycliclic scaffold broadly distributed in plant kingdom (Pratap and Ram, 2014). It has shown a large range of biological activity such as anticancer which was investigated by Lacy and O'kennedy (2004) against two cell linesMCF-7, a breast carcinoma and A549, a lung carcinoma. Anti-inflammatory activity of coumarin derivatives was also determined by the inhibition of carrageenan-induced hind paw edema and the compound was also active in reducing arthritis

induced by Freund's adjuvant (Kontogiordis and Hadjipavlou-Litina, 2005). Antioxidant activity was also observed in coumarins and its derivatives mainly with heterocyclic rings (Fylaktadou *et al.,* 2004). Anti-tubercular activity of phenyl substituted coumarin was reported against *Mycobacterium tuberculosis* (Kawate *et al*., 2013). It was reported that 7-amino 2H-1-benzopyran-2- one derivatives was isolated from some plants such as *Petroselinum crispum*, *Rutagraveolens,Loeselia Mexicana* and*Aesculus pavia*have anti-microbial activity (Navarro-Garcia, 2011).

The extract also contained saturated fatty acid such as heptadecanoic acid which is a C17 saturated fatty acid and is basically a trace component of fats in ruminants. Initially, it was considered to have no biological function but later the increase in circulating levels of these long chained fatty acids have been correlated with an improved insulin sensitivity (Pedersen *et al*., 2016). This further reduces the risk of diabetes type-2 (Forouhi *et al*., 2014) which can be associated with reduced risk of cardiovascular diseases (Khaw *et al*., 2012). In addition, heptadecanoic acid also inhibits cell proliferation in non-small-cell lung cancer clearly showing anticancer activity (Xu *et al*., 2019).

The next compound present in the extract was acacetin which is an abundant flavone found in various plants and some insects. Numerous pharmacological activities have been reported from this class of phytochemicals. Kim *et* al., 2013 studied the antitumor activity of acacetin in which the effects of acacetin was evaluated on prostate cancer in mice and it was found out that acacetin exert antitumor effects by targeting the Akt/NF-κB signaling pathway. Acacetin also exhibited antimicrobial activity in mice against *Staphylococcus aureus* (Bi *et al*., 2016). They demonstrated the protective ability of acacetin from renal abscess formation in mice induced by *S. aureus* thereby increasing the survival rates.

Another long chained fatty acid, 10-Octadecenoic acid, methyl ester was also found in the extract. It was found in the extract of *Iris germanica* also in GC-MS analysis (Asghar *et al*., 2011). It is found to enhance the immunity of hydroxyl unsaturated fatty acid (Yamada *et al*., 2009). 10-Octadecenoic acid, methyl ester was

found to be present in numerous plants extracts including *Thesium humile* (Belakhdar *et al*., 2015) and a well-known medicinal plant *Terminalia arjuna* root (Ramesh and Dhanaraj, 2016).

5.12.2 NMR ANALYSIS

Nuclear magnetic resonance (NMR) is an advance technique for identifying the structure of natural products or compounds and it is an important tool in metabolomic research (Ward *et al*., 2007). It is a suitable method for this analysis as it allows the detection of not only the primary metabolites but also the diverse groups of secondary metabolites simultaneously (Kim *et al*., 2010). ^{1}H NMR spectroscopy is a rapid method ideal for complex sample and it provides huge information without much cost and in less time. In addition, it provides a large quantity of data without previous chromatographic separation proving itself as an ideal technique for the analysis of crude extracts from plants (Pollesello *et al*., 1993). In this study, ^{1}H NMR spectroscopy was used to analyze the structure of the compound which might be responsible for the bioactivity of *Fraxinus floribunda.* After the separation of the extract in column chromatography followed by thin layer chromatographic technique, the extract was subjected to ^{1}H NMR analysis which was compared with the NMR spectra of standard 2-hydrazinoquinoline as it was found to be the most abundant compound revealed through GC-MS analysis. ^{1}H being the most sensitive and commonly occurring magnetic isotope, it is preferred for most of the metabolite fingerprinting and profiling on NMR analysis (Krishnan *et al*., 2005). The ^{1}H NMR spectra of two bands obtained in TLC along with the spectra of standard was compared with the possible spectra with the functional group, it was observed that there were similar sharp peaks at the same region. Moreover, as discussed in result, the similarity in the peaks of spectra of the purified extract with the spectra of the standard, it was established that the purified extract of *F. floribunda* contained 2-hydrazinoquinoline. It is a hydrazine reagent mainly used in the preparation of hydrazone derivatives of carbonyl compounds such as sugar hydrazone have been prepared from it which exhibited antimicrobial activity (Khodair *et al*., 1998). It has also proved as novel derivatization agent for LC-MS based metabolomic investigation of diabetic ketoacidosis. It was demonstrated that 2-

hydrazinoquinoline was compatible with biological samples such as urine, serum, liver extract samples and by using this approach, it was convenient to characterize the kinetics in diabetes-induced (type1) metabolic changes in streptozotocin-treated mice (Lu *et al*., 2013). Hydrazone derivatives are known for various biological activities which we have discussed above on GC-MS analysis. In this study, the NMR analysis was done to focus on the antidiabetic activity of the *F. floribunda* bark extract. In the study reported by Smalley *et al*., (2006), a set of novel heterocyclic pyrimidyl hydrazones could be used as antidiabetic agent by inhibiting glycogen synthase kinase-3 (GSK-3). GSK-3 is a protein which serves as many functions in the human body, one of which is to mediate the conversion of glycogen to glucose. The diabetic patients with insulin resistance have GSK-3 activity in their body and it leads to rise in plasma glucose level in hyperglycemia. Thus, these hydrazone derivatives serve as GSK-3 inhibitors therefore by serving as antidiabetic agents in hyperglycemic condition.

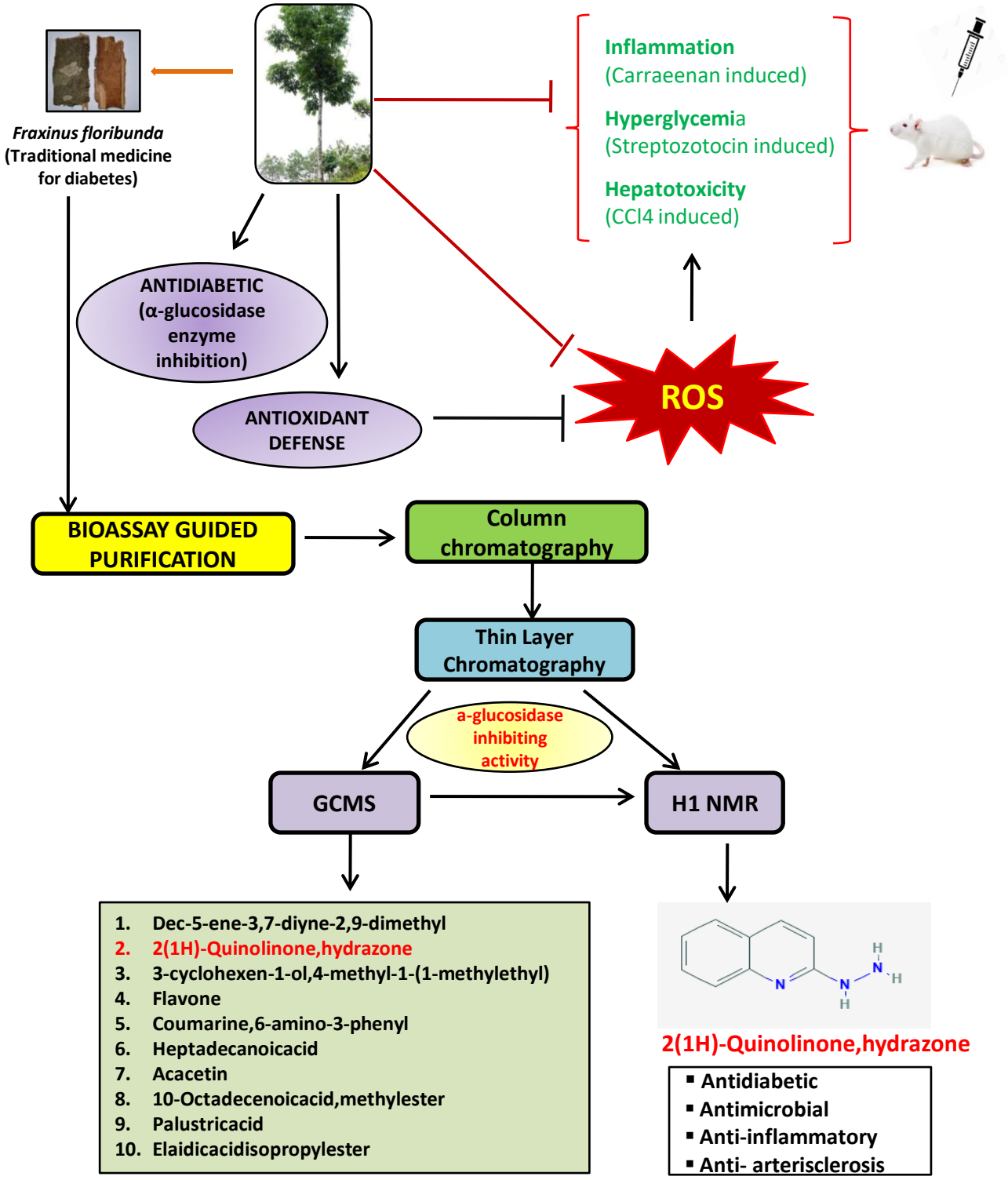

Figure 5: A flowchart of study of ethnomedicine from traditional system to the identification of bioactive compound responsible for its bioactivity.