Comparison of Arjuna ghritas prepared from physiologically shaded and marketed Arjuna bark for its cardio protective activity

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Submitted for the partial fulfillment of the requirements for the Degree of

Master of Pharmacy in **Pharmacognosy**

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Rashtrasant Tukadoji Maharaj Nagpur University,

Nagpur, Maharashtra, India

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Certificate

This is to certify that, investigations described in this thesis entitled,

"Comparison of Arjuna Ghritas Prepared from Physiologically Shaded and Marketed Arjuna Bark For its Cardio Protective Activity"

were carried out by **Ms. Bharati S. Mazi** in the laboratories of the Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, under my supervision and guidance, for the partial fulfillment of the requirements for the degree of **Master of Pharmacy** in **Pharmacognosy** in the **Faculty of Science and Technology**, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur.

The thesis is now ready for examination.

Date:

Place: Nagpur

Dr. Satyandra K. Prasad
(Guide)

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TO WHOMSOEVER IT MAY CONCERN

This to certify that Ms. Bharati S. Mazi studying in University Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur - 440 033, Pharmacy – Fourth semester has completed her project work on **"Comparison of Arjuna ghritas prepared from physiologically shaded and marketed *Arjuna bark* for its cardio protective activity".**

The duration of project work was ten months i.e. 1st August, 2019 to 31st May 2020. Now she has completed her dissertation work. The above research project work has been completed jointly under the collaboration of **University Department of Pharmaceutical Sciences Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur and Go-Vigyan Anusandhan Kendra, Deolapar, Nagpur, Maharashtra.**

During this Period, she was sincere in her work.

We wish her all the success in future.

Yours Sincerely

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Declaration

*I hereby declare that the thesis entitled "**Comparison of Arjuna Ghritas Prepared from Physiologically Shaded and Marketed Arjuna Bark for its Cardio Protective Activity**"*

*is an original research work carried out under the supervision of **Dr. Satyendra K. Prasad** in the laboratories of Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur. This work has not been submitted in part or full for any degree or diploma of any other University.*

Place: Nagpur *Bharati S Mazi*

Date:

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"One moment can change a day, one day can change a life and one life can change the world"

- Buddha

To commence with, I pay my obeisance to GOD, the almighty to have bestowed upon me good health, courage, inspiration, zeal and the light. After GOD, I express my sincere and deepest gratitude and indebtness to my supervisor, ***Dr. Satyendra K. Prasad sir*** *for his unstinting guidance, myriad favor, continued assistance and encouragement and these all have shape me in such a way that I was able to construct this thesis smoothly. I really admire his professional ethics and profound dedication, which is difficult to express in words. I feel very much honored and it has been a real privilege for me to get an opportunity to work under his guidance.*

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Date:

Place: Nagpur ***(Bharati S Mazi)***

Dedicated to My

Parents,

Family & Friends

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ABBREVIATIONS AND SYMBOLS

% – Percentage	**Kg- Kilogram**
° – Degree	**mg-Milligram**
sec – Second	**mL- Millilitres**
µg – Microgram	**mm- millimetre**
µl– Microliter	**nm- Nanometre**
µm– Micrometer	**NaOH– Sodium hydroxide**
µM– Micromolar.	**R_{f-} Retention Factor**
cm – Centimeter	**rpm - Revolutions per minute**
^{0}C- Degree Celsius	**STD- Standard**
FCR-Folin Ciocalteau reagent	**TLC-Thin Layer Chromato- graphy**
H_2SO_4 – Sulphuric acid	**UV-Ultraviolet**
HCl–Hydrochloric Acid	**v/v– volume/volume**
HNO_3 – Nitric acid	**w/w– weight/weight**
IAEC-Institutional Animal Ethics Committee	**w/v– weight/volume**
g-Gram	**LPO- Lipid peroxidation**
IP-Indian Pharmacopoeia	**USP-United state Pharmacopoeia**
r.p.m-Rotations per minute	**HFD-High Fat Diet**
LDL-Low density Lipoprotein	**HDL-High density Lipoprotein**
TG- Triglycerides	**VLDL-Very low desnsity lipoprotein**
Std-Standard	**Inhouse LD- Inhouse low dose**

Inhouse HD- Inhouse high dose	**Mark LD- Marketed low dose**
Mark HD-Marketed high dose	**CVD-Cardiovascular diseases**
MI-Myocardial Infraction	**CAD-Coronary artery disease**
ALT-Alanine transaminase	**AST-Aspartate transaminase**
DM-Diabetes mellitus	**min-Minutes**

INTRODUCTION……..

1. CARDIOVASCULAR DISEASE:

CVD is a class of diseases which are related to the heart or blood vessels including stroke, heart failure, hypertension, coronary artery diseases, heart arrhythmia, peripheral artery disease, and atherosclerosis. Individuals with CVD are found to have been accompanied with issues like raised blood pressure, elevated glucose, smoking, obesity, lack of exercise, excessive alcohol consumption, and dyslipidemia. Fortunately, CVD can be properly managed and prevented by controlling blood pressure, glucose, lipid, smoking, and alcohol drinking and through lifestyle modifications for sleep, emotion, exercise, and diet, which are called SEED intervention .With the aging of population in the world, CVD has become the leading cause of death globally. Approximately 17.9 million deaths in 2015 were caused by CVD in the world. The percentage of Chinese older than 60 has increased to 16% in 2015 which directly leads to a consequence that cardiovascular disease is becoming the leading cause of death in China. Multiple risk factors contributing to CVD include obesity, high blood pressure, diabetes, aging, male sex, metabolic syndrome, and physical inactivity. Pharmacologic treatment of CVD in conjunction with therapeutic lifestyle changes can be used for both primary and secondary prevention of cardiovascular disease.

CVD comprises many different types of condition. Some of these might develop at the same time or lead to other conditions or diseases within the group.

Diseases and conditions that affect the heart include:

- **Angina**, a type of chest pain that occurs due to decreased blood flow into the heart
- **Arrhythmia**, or an irregular heartbeat or heart rhythm
- Congenital heart disease, in which a problem with heart function or structure is present from birth
- **Coronary artery disease**, which affects the arteries that feed the heart muscle
- **Heart attack**, or a sudden blockage to the heart’s blood flow and oxygen supply
- **Heart failure**, wherein the heart cannot contract or relax normally
- **Dilated cardiomyopathy**, a type of heart failure, in which the heart gets larger and cannot pump blood efficiently

- **Hypertrophic cardiomyopathy**, in which the heart muscle walls thicken and problems with relaxation of the muscle, blood flow, and electrical instability develop
- **Mitral regurgitation**, in which blood leaks back through the mitral valve of the heart during contractions
- **Mitral valve prolapse**, in which part of the mitral valve bulges into the left atrium of the heart while it contracts, causing mitral regurgitation
- **Pulmonary stenosis**, in which a narrowing of the pulmonary artery reduces blood flow from the right ventricle (pumping chamber to the lungs) to the pulmonary artery (blood vessel that carries deoxygenated blood to the lungs)
- **Aortic stenosis**, a narrowing of the heart valve that can cause blockage to blood flow leaving the heart
- **Atrial fibrillation**, an irregular rhythm that can increase the risk of stroke
- **Rheumatic heart disease**, a complication of strep throat that causes inflammation in the heart and which can affect the function of heart valves
- **Radiation heart disease**, wherein radiation to the chest can lead to damage to the heart valves and blood vessels

Vascular diseases affect the arteries, veins, or capillaries throughout the body and around the heart.

They include:

- Pperipheral artery disease, which causes arteries to become narrow and reduces blood flow to the limbs
- Aneurysm, a bulge or enlargement in an artery that can rupture and bleed
- **Atherosclerosis**, in which plaque forms along the walls of blood vessels, narrowing them and restricting the flow of oxygen rich blood
- Renal artery disease, which affects the flow of blood to and from the kidneys and can lead to high blood pressure
- Raynaud’s disease, which causes arteries to spasm and temporarily restrict blood flow
- Peripheral venous disease, or general damage in the veins that transport blood from the feet and arms back to the heart, which causes leg swelling and varicose veins

- **Ischemic stroke**, in which a blood clot moves to the brain and causes damage
- **Venous blood clots**, which can break loose and become dangerous if they travel to the pulmonary artery
- **Blood clotting disorders**, in which blood clots form too quickly or not quickly enough and lead to excessive bleeding or clotting
- **Buerger's disease,** which leads to blood clots and inflammation, often in the legs, and which may result in gangrene

It is possible to manage some health conditions within CVD by making lifestyle changes, but some conditions may be life threatening and require emergency surgery.(Medical News Paper, reviewed by Dr.Payal Kohli, July 2019)

1.1 Pathophysiology of CVD: (Edgardo Ol a Lopez et al,. Aug 10, 2020) ver

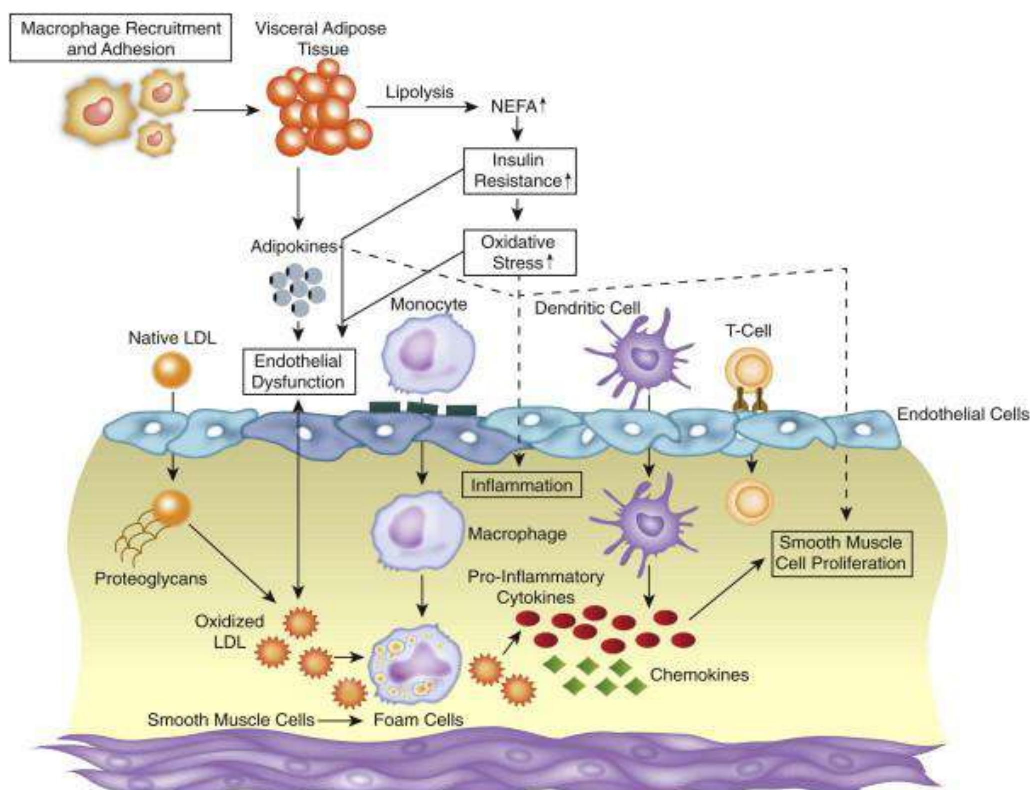


Fig 1: Pathophysiology of CVD

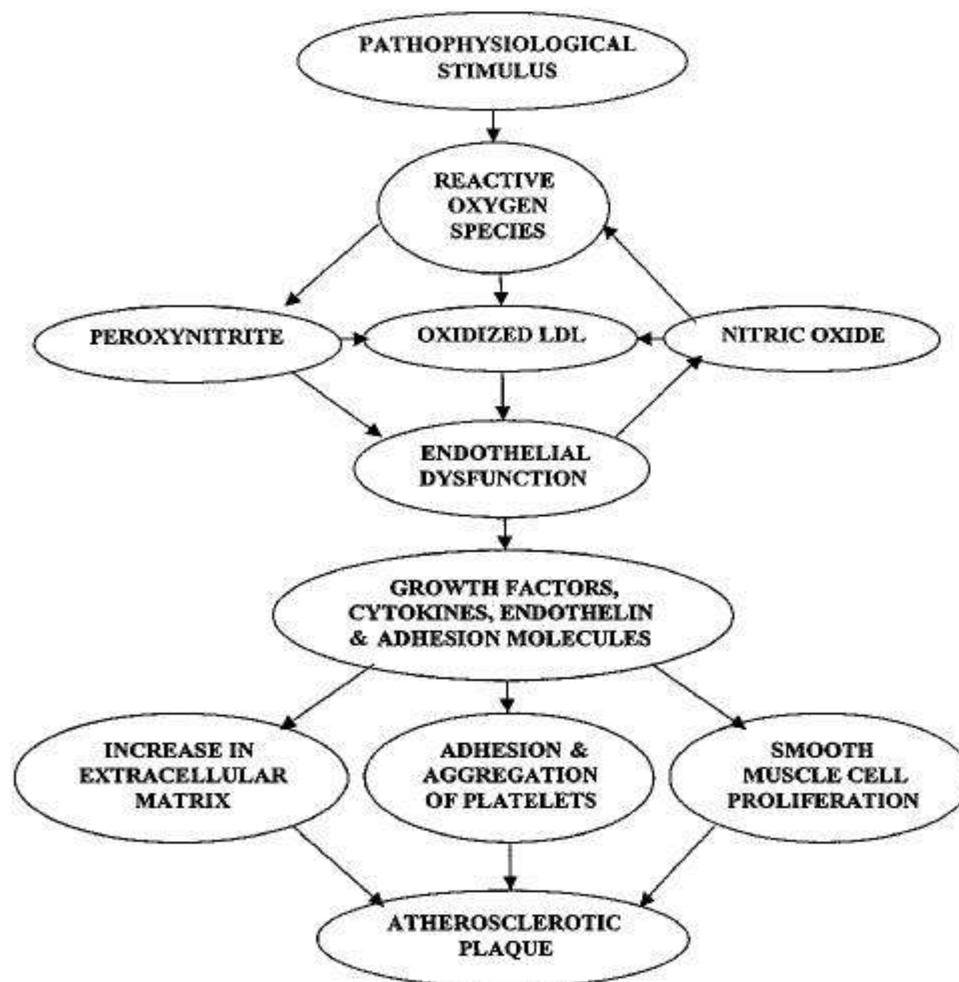


Fig 2: Schematic diagram depicted the involvement of reactive oxygen species, endothelial dysfunction, growth factor, cytokines and adhesion molecules in the genesis of atherosclerosis.

1.3 Treatment of CVD in herbal: Arjuna Ghrita

1.3.1 Arjuna bark

Arjuna (Roxb) Wight & Arn. , family Combretaceae is a miracle herb which was used during ancient times to cure heart problems. In ancient Ayurvedic literature, Vagbhata and others have described the juice of Arjuna bark as a tonic and astringent. They have recommended it for the treatment of heart diseases. Arjuna is reported to be a beneficial herb in treating heart problems since 1200 B.C. Vagbhatta was the first to cite this in his book "Astang Hridayam" written some 1200 years ago. Subsequently, Chakradutta and also Bhawa Mishra described its use in chest pain. Several medicinal plants have been described to be beneficial for cardiac ailments in Atharva Veda an ancient treatise from which Ayurveda, the

Indian system of Medicine owes its origin. Ancient medical scientists have mentioned the properties of *Terminalia arjuna* (Roxb) Wight & Arn. herb. Indian medical knowledge known as Ayurveda goes back millennia. The Vedas and Puranas refer various materials of medical importance including herbs, plants and trees. In which *Terminalia arjuna* (Roxb) Wight & Arn. bark (arjuna) has prominent place and have been identified and researched for their putative lipid lowering and cardio protective activities. The plant which has shown most promising and distinct results is *Terminalia arjuna* (Roxb) Wight & Arn. bark popularly known as arjuna. Modern research has discovered that *Terminalia arjuna* (Roxb) Wight & Arn. has antioxidant properties and may be clinically helpful in cardiovascular health.

Most of the studies, both experimental and clinical, have suggested that the crude drug possesses anti-ischemic, antioxidant, hypolipidemic, and antiatherogenic activities. Its useful phytoconstituents are: Triterpenoids, β-sitosterol, flavonoids, and glycosides. Triterpenoids and flavonoids are considered to be responsible for its beneficial antioxidant cardiovascular properties. The drug has shown promising effect on ischemic cardiomyopathy. So far, no serious side effects have been reported with *arjuna* therapy. However, its long-term safety still remains to be elucidated. Though it has been found quite useful in angina pectoris, mild hypertension, and dyslipidemia, its exact role in primary/secondary coronary prevention is yet to be explored. (Shridhar Dwivedi and Deepti Chopra, 2014)

1.3.2 Ghrita

Ghrita (medicated ghee) is the Ayurvedic medicinal preparation in which ghee is processed with some Kashaya (herbal decoctions) and kalka (fresh paste) of herbs. The choice of decoctions and paste of herbs are based on the formula mentioned in the Ayurvedic texts or Ayurvedic formulary of India.

Ghrita or ghee is a type of fat (sneha dravya); hence the fat-soluble active principles of the ingredients are properly dissolved in Ghee and ensure their absorption in the body. Also, only ghee is the medium, which crosses the blood-brain barrier, the drugs indicated for brain and nervous system disorders, when processed in ghee and used; acts best which no other dosage form can.(Anjali Darevey at el,.2016)

1.3.3 Method of Preparation of Ghrita (medicated ghee):

Before going to start the method of preparation of Ghrita, you should know some basics about Ghrita or Sneha kalpana.

In the preparation of medicated ghee, there are three essential components. These are Drava, Kalka and Sneha dravya.

- Drava is a liquid, which may be one or more in number as mentioned in the formula. This may be Kashaya (decoction), Swarasa (fresh juice extracted from crushed leaves or whole herb), Dugdha (milk), Mastu (curd water), Mamsa Rasa (meat soup) etc.
- Kalka is a fine paste of one or more drugs (either fresh or dried).
- Sneha dravya is the base material. Ghrita (ghee) and Taila (oil) are used for this purpose, either one or in combination (yamaka).

When the quantities of these ingredients are not mentioned in the formula then we follow some general rules described in Ayurvedic classics. These rules are –

- If no Drava (liquid) is prescribed, the Kalka is 1 part by weight, Sneha Dravya should be 4 parts and 16 parts of water are added in the place of Dravya.
- If Drava dravya (liquid) is Kwatha or Kashaya (decoction), 1 part Kalka and 6 parts Sneha dravya is taken.
- If the Drava dravya (liquid) is Swarasa (juice), Ksheera (milk), Takra (butter milk), Dadhi (curd) etc., 1 part Kalka, 8 parts Sneha Dravya is taken. 16 times water is also added to ensure proper processing and dissolution of active ingredients of drugs.
- If there are four or less than four number of Drava dravya (liquid) is mentioned in the formula, each Drava dravya (liquid) should be taken four times the weight of Sneha.
- If the Drava dravya (liquids) are 5 or more in number, each Drava (liquid) should be taken equal in weight to the Sneha.
- If there is no Kalka prescribed in the formula, then the drugs of the Kashaya (decoction) may be used as Kalka.

1.3.4 How to prepare Ghrita (medicated ghee):

Generally following steps are followed in Ghrita preparation:

The Kalka and the Drava mentioned in the formula are first mixed together in a vessel.

Sneha dravya (ghee) is then added and boiled on mild fire. It is stirred well continuously so that the Kalka (solid part of the mixture) should not adhere to vessel.

There is another method described in Ayurvedic text about adding liquids. The Drava/ dravyas (liquids) are directed to be added one after another when the previously added Drava

(liquid) has evaporated as the process of the boiling is continued. After a time span, the moisture content in the Kalka will begin to evaporate after when all the Drava dravyas (liquids) have evaporated. At this stage, we should take extra care to stir more often and carefully to ensure the Kalka should not be adhering to the vessel's bottom. The small amount of Kalka is taken out time to time during the process with the help of a ladle and tested to know the condition and stage of the Paka.

There are three stages of Paka –

1. Mridu Paka – In this stage, the Kalka is waxy in consistency and when rolled between the fingers, rolls like lac with slight sticking. When the rolled Kalka is put in fire, it burns with a cracking sound. It is used for Nasya Karma.
2. Madhyama Paka – In this stage, the Kalka is soft, non-sticky and rolls between fingers. When it is put on fire, it burns without any cracking sound. It is used for Pana (Abhyantara sneha pan/ internal oleation therapy or oral intake) and Vasti Karma (therapeutic enema).
3. Khara Paka – The next degree of heating is Khara paka, which is slightly hard. It is used only for Abhyanga (external oleation therapy/ massage).
4. Furthermore, heating will lead to Dagdha Paka and the Sneha becomes unfit for use.
5. After gaining the required stage of paka, the heating is stopped and the final product is kept for cooling naturally.(Dr Maish Tomar, Aug 27,2014)

1.4 Arjuna ghrita (Kshal Nikode and Vaishali Kuchewar, Original research article)

Arjuna Ghrita is an herbal ayurvedic ghee that is prepared by the Arjun bark as well as cow's milk. Being made up of these two ingredients, Arjun ghrit is considered to be very beneficial for dealing with the heart related conditions.

Arjun bark is the major ingredient used to formulate Arjun Ghrita. It acts like a cardiac tonic; therefore, it can be effectively used to treat heart and cardiovascular diseases. Arjun Ghrita is capable of preventing the plaques accumulation in the arteries thereby it makes you less receptive to heart and cardiovascular issues.

1.4.1 Ingredient of Arjuna ghrita :

Partha svarasa (Arjuna) (St.Bk.) 16 parts

Ghrita (Go-ghrita) 4 parts

Partha kalka (St.Bk.) 1 parts

1.5 Health benefits of Arjuna ghrita

- Angina
- Herat palpitation
- Cardiac tonic
- Bleeding disorders
- Breathlessness
- Antioxidants activity
- It cures internal dryness.
- It improves weight, lustre, and digestion.

LITERATURE REVIEW…….

2. LITERATURE REVIEW:

- Savita et al (2015). Gives a comparative study on antioxidant activity of two formulations which are Partha Aristha and Arjuna Ghrita containing *Terminalia Arjuna* found that the formulations having confirmed antioxidant activity.
- Gopi (2017).Explains the various medicinal effects which majorly include various heart diseases such as cardiac pain, atherosclerosis, CHF, high blood pressure level, high cholesterol level. Also reveals that it is an anti-ischemic agent, antioxidants etc.
- Dwivedi and Chopra (2014). They revisite about ancient cardiovascular activity of drug Arjuna. Most of the studies explain the activity of arjun in various heart conditions.
- Daverey et al 2016. They give about the Shelf Life Estimation of Ayurvedic Cardiotonic: Arjuna Ghrita – The literature reveals about the shelf life the formulation which should be up to 6 months to 2 years. The paper also reveals that during this period the organoleptic characters of Arjuna ghrita remains the same but the physicochemical variables shows a degree of changes at each interval of 15, 30, 60, 90 & 180 days.
- Anupama 2013. Arjun Ghrita for heart diseases – from the article it is found that the formulation of Arjuna as Arjuna ghrita having potent action on various heart diseases includes angina, heart bloating, palpitation, bleeding disorders, bloating etc.

- Methanolic extract of Arjuna ghrita demonstrated effective hypocholesterolmic action together with antioxidant effect in rats. Antihyperlipidemic activity was determined using cholesterol suspension induced hypercholesterolemia in rats. Rats were randomly checked. Cholesterol suspension and test drugs were administered for period of 28 days. At the end of experimental period blood was withdrawn from retro-orbital plexus and was used for biochemical estimation. Liver was analysed for antioxidant parameters. Administration of ethanolic extract of Arjuna ghrita significantly decreased serum TC, TG, LDL-C, VLDL-C levels, atherogenic indices and significantly increased serum HDL-C level. Lipid peroxidation was significantly decreased whereas reduced glutathione and catalase levels were significantly increased in liver (Dhande et al., 2014).
- The effect of Arjuna ghrita on lipid profile after high cholesterol diet for 45 days was conducted on healthy rabbits of either sex. Biochemical tests were performed at the

completion of dosing. Arjuna ghrita revealed a significant reduction in serum cholesterol, triglycerides and low density lipoprotein levels (Razzaq et al., 2011).

- Lipid lowering activity of a methanolic extracts of Arjuna ghrita was elevated in guinea pigs fed with a high cholesterol diet. Serum lipid profile (total cholesterol, triglycerides, LDL-C, VLDL-C and HDL-C) and serum enzymes (ALT, AST, ALP, LDH and CK-MB) were performed in each group at 0 days and at the end of 60 days. Histological study of liver and kidney was done. It showed lipid lowering activity and the higher doses of it was found to reduce serum AST, ALP and LDH levels. Histology of the liver shows decreased lipid accumulation. (Chawda et al., 2014).
- Various studies showed that consumption of 10% of ghee in daily routine dose not affects the health condition. The experiment performed on Fischer inbred rats indicates that consumption of 10% ghee may increase triglyceride levels, but does not increase lipid peroxidation processes that are linked to a higher risk of cardiovascular disease. Many research studies have been published, which report beneficial properties of ghee and herbal mixtures containing ghee. In animal studies, there was a dose-dependent decrease in serum total cholesterol, LDL, VLDL, and triglycerides; decreased liver total cholesterol, triglycerides, and cholesterol esters; and a lower level of nonenzymatic-induced lipid peroxidation in liver homogenate, in Wistar outbred rats. Similar results were obtained with heated (oxidized) ghee. When ghee was used as the sole source of fat at a 10% level, there was a large increase in oleic acid levels and a large decrease in arachidonic acid levels in serum lipids. In rats fed ghee-supplemented diets, there was a significant increase in the biliary excretion of cholesterol with no effect on the HMG CoA reductase activity in liver microsomes. A 10% ghee-supplemented diet decreased arachidonic acid levels in macrophage phospholipids in a dose-dependent manner. Serum thromboxane and prostaglandin levels were significantly decreased and secretion of leukotrienes by activated peritoneal macrophages was significantly decreased. (Hari Sharma et al,.2010)
- The lipid lowering action of the bark powder of *Tenninalia arjuna* (I: arjuna) has been studied in triton and cholesterol fed rats. Serum lipids were found to be lowered by I: arjuna (100mg/kg, b.w.) in triton induced hyperlipaemia. Chronic feeding of this powder (100mg/kg, b.w., p.0.) in animals simultaneously fed with cholesterol (25 mgkg, b.w.1 for 30 days, caused lowering in lipids and protein levels of lipoproteins followed by an increase in high density lipoprotein-cholesterol compared with the cholesterol fed groups. I: arjuna alters lipolytic activities in plasma, liver, heart and

adipose tissues of hyperlipaemic rats. The lipid lowering action of this natural product is mediated through inhibition of hepatic cholesterol biosynthesis, increased faecal bile acid excretion and enhanced plasma 1ecithin: cholesterol acyltransferasea activity and stimulation of receptor mediated catabolism of low density lipoprotein.

- Ghrita consist of various fats such as saturated, monosaturated and polyunsaturated, fatty acids, minerals, vitamins etc. And the composition of Ghrita varies along with the method of preparation. Scientific researches carried out on Ghrita plain as well as medicated have reported about the depressant effects of medicated Ghrita in gross behavioural tests, potentiated phentobarbitone sleeping time, analgesic effect and stimulatory effect on cognition. Further, studies conducted to evaluate the effect of Ghrita on the serum lipid levels showed a dose dependant decrease in the total cholesterol, low density lipoproteins, and very low density lipoproteins. Ghrita was also reported to have wound healing activity. (Vd. Varnika Singh at el., 2019).
- Ghee (G) is attributed with numerous health benefits in Ayurveda. However, due to the high saturated fat content, it has been predicted to increase the cardiovascular disease risk. Hence, the current study was performed to evaluate the effect of G consumption as compared to mustard oil (MO) on lipid profile. When study conducted using two hundred (100 males) apparently healthy adults (≥40 years) were randomly selected out of the total individuals interviewed in a house-to-house survey. They were divided into three groups based on G and MO consumption: (A) MO >1 L/month, G<0.5 kg/month; (B) MO 1–0.5 L/month, G 1.25–0.5 kg/month; and (C) MO <0.5–0.2 L/month, G>1.25 kg/month. Serum lipid parameters were compared among the groups. It was found that the group C had the significantly lowest triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), TC/HDL and LDL/HDL and highest high-density lipoprotein (HDL).
- Ghee, the anhydrous milk fat, is one of the most important sources of dietary fat in India. Male Wistar rats were fed diets containing 2.5, 5.0 and 10 wt% ghee for a period of 8 weeks. The diets were made isocaloric with groundnut oil. The results showed that serum thromboxane levels decreased by 27–35%, and 6-keto-prostaglandin $F_{1\alpha}$by 23–37% when ghee was incorporated at level of 10% in the diet. Prostaglandin E_2levels in serum and secretion of leukotrienes $B_4C_4$and $D_4$by peritoneal macrophages activated with calcium ionophore decreased when increased

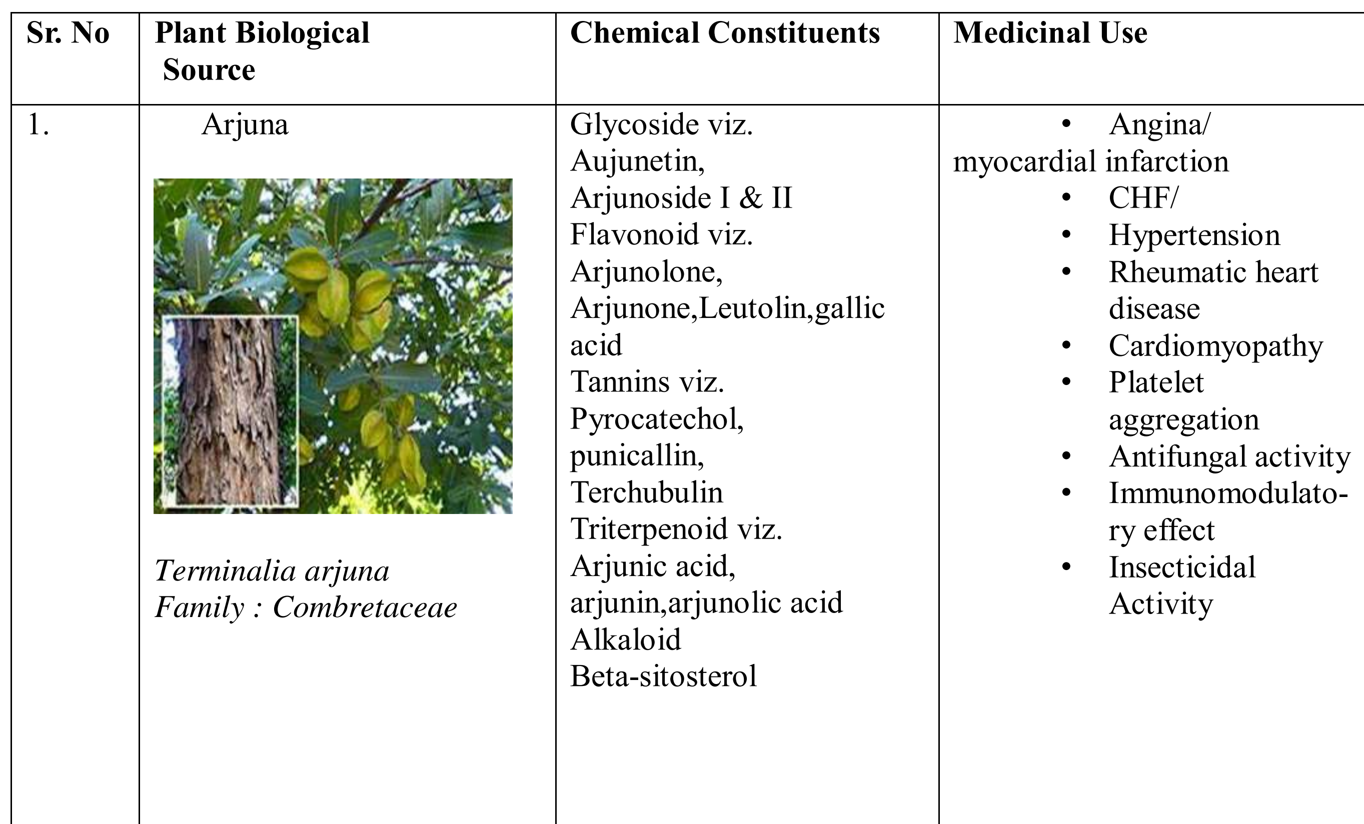
amounts of ghee from 2.5 to 10% were included in the diet. Arachidonic acid levels in macrophage phospholipids decreased when incremental amounts of ghee were fed to rats. These studies indicate that ghee in the diet not only lowers the prostaglandin levels in serum but also decreases the secretion of leukotrienes by macrophages.(M.Vijay Kumar at el., 1999)

- To determine the association between intake of dietary fat, specifically Indian ghee, and prevalence of coronary heart disease (CHD) and risk factors as study was undertaken on a rural population in Rajasthan. Total community cross-sectional survey was done using a physician administered questionnaire; 1982 males aged 20 years and more were studied. The dietary questionnaire focused on the amount and type of fat consumed. Staple dietary fat in this area is mustard/rapeseed oil and Indian ghee. To define the role of ghee, the average amount consumed in a month was determined; 782 males (39%) consumed 1 kg or more ghee per month (group 1) and 1200 (61%) consumed less than 1 kg per month (group 2). To elicit details of fatty acid composition of the diet consumed, detailed dietary history was acquired from a random 460 (23%) males; 220 from group 1 and 240 from group 2. Group 1 males were significantly younger, more literate and had more weight and body-mass index. This group consumed significantly more calories, saturated and mono-unsaturated fats while the consumption of polyunsaturated fats was similar in the two groups. Fatty acid intake analysis showed that group 1 males consumed more mono-unsaturated (n-9) fatty acids than group 2. Intake of polyunsaturated n-3 and n-6 fatty acids was similar. There was significantly lower prevalence of CHD in men who consumed > kg ghee per month (odds ratio = 0.23, 95% confidence limits 0.18-0.30, $p < 0.001$). Multivariate analysis confirmed this association ($p < 0.001$). The prevalence of hypertension and other coronary risk factors was similar in the two groups.
- The bark of the tree *Terminalia arjuna* (Roxb.) is widely used in Indian medicine (*Ayurveda*) for various cardiovascular ailments. The bark has been reported to contain several bioactive compounds. Many experimental studies have reported its antioxidant, anti-ischemic, antihypertensive, and antihypertrophic effects, which have relevance to its therapeutic potential in cardiovascular diseases in humans. Several clinical studies have reported its efficacy mostly in patients with ischemic heart disease, hypertension, and heart failure. However, a major shortcoming in all these experimental and clinical studies is the absence of phytochemical standardization of the extracts. (Subir K. Maulik and Kewal K. Talwar 2012)

PLANT PROFILE.......

<u>3.PLANT PROFILE :</u> ***Teminalia arjuna***

<u>Table No 1: Plant Profile</u>

Sr. No	Plant Biological Source	Chemical Constituents	Medicinal Use
1.	Arjuna  *Terminalia arjuna* *Family : Combretaceae*	Glycoside viz. Aujunetin, Arjunoside I & II Flavonoid viz. Arjunolone, Arjunone,Leutolin,gallic acid Tannins viz. Pyrocatechol, punicallin, Terchubulin Triterpenoid viz. Arjunic acid, arjunin,arjunolic acid Alkaloid Beta-sitosterol	• Angina/ myocardial infarction • CHF/ • Hypertension • Rheumatic heart disease • Cardiomyopathy • Platelet aggregation • Antifungal activity • Immunomodulato-ry effect • Insecticidal Activity

4.1 Habitat:

Arjuna tree is about 60-80 ft. in height, and is seen along rivers, streams and dry water bodies throughout the Indo-sub-Himalayan tracts of Uttar Pradesh, southern Bihar, Chota Nagpur, Burma, Madhya Pradesh, Delhi, and Deccan region. It is also found in the forests of Sri Lanka and Mauritius**.** It grows almost in all types of soils, but prefers humid, fertile loam and red lateritic soils. It can tolerate half submergence for a few weeks. Arjuna is propagated by seeds; Germination takes 50-70 days with 50-60% germination. (.(Shridhar Diviwedi and Deepti Chopra, 2014)

Fig 3: *Terminalia arjuna* tree

Fig 4: Stem bark of *Terminalia arjuna*

fig 5: Bark pices of *Terminalia arjuna*

4.2. Pharmacognostic feature:

The outer surface of the bark is smooth, while the inner surface has longitudinal striation and is pinkish in colour**.** The bark gets flaked off itself in the month of April–May. On microscopic examination of the mature bark, a cork consisting of 9-10 layers of tangentially elongated cells, 2-4 cells thick phellogen, and phelloderm consisting of tangentially elongated cells are seen. The phloem is broad, consisting of ceratenchyma, phloem parenchyma, phloem fibres, and crystal fibres with rosette crystals of calcium oxalate. Periderm and secondary phloem are present in the old bark. Leaves are sub-opposite, coriaceous, oblong/elliptic, dull green from the upper side and pale brown on the lower side, often unequal sided with 10-15 pairs of nerves. Flowers are white in colour and bisexual, arranged in spikes with linear bracteoles. Fruits are ovoid/oblong with 5-7 hard angles or wings. The lines on wings are oblique and curving upward. Major chemical constituents of arjuna have been shown in various extracts of the stem bark of arjuna have shown to possess many pharmacological properties including inotropic, anti-ischemic, antioxidant, blood pressure lowering, antiplatelet, hypolipidemic, antiatherogenic, and anti-hypertrophic**.** Thus, in the present article, we have made an attempt to review and give up-to-date information pertinent to the usage of Arjuna as a potential cardio protective agent. (Vinod Dhingra el al,. March 2013)

4.3. Phytoconstituents:

Table No 2: Phytoconstituents

PART OF PLANT	PHYTOCONSTITUENTS
Stem bark	Terpenoids Glycosides Flavonoids Tannins β-setosterole mineral/trace elements

Roots	Triterpenoids Glycosides β-setosterole
Leaves	Flavonoids Alkaloids Tannins Phenolic compound Oxalic acid Inorganic acid
Fruits	Glycoside Flavonoids
Seed	Cartenolide

4.4 Clinical uses of Arjuna:

- Hypolipidemic and antiatherogenic activity.
- Angina/myocardial infarction.
- CHF/hypertension.
- Rheumatic heart disease.
- Ischemic mitral regurgitation.
- Thrombotic condition behavioural changes.
- Immunomodulatory, antioxidant and anti-inflammatory.(Shridhar Diviwedi and Deepti Chopra, 2014)

OBJECTIVE…….

4. OBJECTIVE:

The main objective of the study is to perform a comparative phytochemical, chromatographic and preclinical evaluations on Arjuna Ghrita prepared from physiologically shaded and marketed Arjuna Bark for its cardio protective activity and present the evaluated data for the future studies.

PLAN OF WORK……..

5. PLAN OF WORK

1. Collection and authentication of Arjuna bark.
2. Collection and authentication of other raw materials.
3. Macroscopical and microscopical evaluation.
4. Extraction of plant material.
5. Qualitative physicochemical evaluation.
6. Quantitative phytochemical estimation.
7. Chromatographic evaluation preparation of Arjuna ghrita.
8. Pharmacological evaluation for cardio protective activity.
9. Histopathology and biochemical evaluations.

MATERIAL & METHOD………

6. MATERIAL AND METHOD

6.1 Formulation Table:

Table No 3: Formulation Table

SR NO	INGREDIENTS	WEIGHT(g and ml)
1	Partha svarasa (Arjuna) (St.Bk.)	250
2	Ghrita	1000
3	Partha Kalka (St.Bk.)	8000

6.2 Procurement of marketed Arjuna Ghrita formulation:

The marketed ghrita formulation and raw material for preparation of in-house ghrita formulation were taken from “Go-Vigyan Kendra.”

6.3 Authentication of plants in formulation

The plant material was authenticated by Dr. Prakash R. Itankar, Associated Professor, Dr. Satyendra K. Prasad, Assistant Professor, Pharmacognosy Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur.

Table No 4: Authentication Table

Sr. No.	Common Name	Botanical Name
1.	Arjuna	*Terminalia arjuna*

6.4 Preparation of In-house Arjuna Ghrita formulation:

The Kalka and the Drava mentioned in the formula had first mixed together in a vessel. Then after, sneha dravya (ghee) had added and boiled on mild fire. It has then stirred well continuously so that the Kalka (solid part of the mixture) should not adhere to vessel.

There is another method described in Ayurvedic text about adding liquids.

The Drava/ dravyas (liquids) are directed to be added one after another when the previously added Drava (liquid) gets evaporated as the process of the boiling is continued. After a time span, the moisture content in the Kalka will begin to evaporate. At this stage, we should take extra care to stir more often and carefully to ensure the Kalka should not be adhering to the vessel's bottom. The small amount of Kalka is taken out time to time during the process with the help of a ladle and tested to know the condition and stage of the Paka.

6.5 Organoleptic Evaluation:

Macroscopic evaluation of Arjuna Ghrita formulation was done on the basis of its physical properties such as state, colour, odour, taste and texture.

6.6 Phytochemical screening: (Khandelwal et al,. 2007)

The plant may be considered as biosynthetic laboratory for multitude of compounds like alkaloids, glycosides, volatile oils, tannins, saponins, flavonoids, proteins, steroids, phenols etc. These compounds are termed as secondary metabolites and are responsible for therapeutic effects.

To check the presence or absence of primary and secondary metabolite, the formulation were subjected to phytochemical screening.

6.6.1 Test for Sterols:

A) Salkowski test:

Few mg of extract of each extract was taken in 2 ml of chloroform and in it 2 ml of concentrated sulphuric acid was added from the side of the test tube. The test tube was shaken for few minutes. The development of red colour in the chloroform layer indicates the presence of sterols.

B) Liebermann-Burchard test:

Few mg of extract was dissolved in chloroform. To this, few ml of acetic anhydride was added. Boil for few seconds and cool. Then two drops of concentrated sulphuric acid were added from the side of the test tube. Brown ring indicates the presence of sterols.

6.6.2 Test for Alkaloid:

Few mg of the residue of each extract was taken separately in 5 ml of 1.5 % v/v hydrochloric acid and filtered. These filtrates were then used for alkaloid detection.

A) Dragendroff's reagent:

It was prepared by mixing solution A (17 g of bismuth sub nitrate + 200 g of tartaric acid + 800 ml distilled water) and solution B (160 g potassium iodide + 400 ml distilled water). Above Dragendroff's reagent was sprayed on Whatmann No. 1 filter paper then the paper was dried. The test filtrate after basification with dilute ammonia was extracted with chloroform and this extract was applied on the filter paper, impregnated with dragendroff's reagent, with the help of capillary tube. Development of an orange red colour on the paper indicates the presence of alkaloids.

B) Mayer's Reagent:

1.36 g of mercuric chloride was dissolved in 60 ml water and 5 g of potassium iodide dissolved in 10 ml of distilled water, solution were mixed and diluted to make up volume 100 ml. To a little of each extract taken in dilute hydrochloric acid in a watch glass, few drops of the reagent was added, formation of cream colored precipitate shows the presence of alkaloids.

C) Wagner's Reagent:

1.27 g of iodine and 2 g of potassium iodide were dissolved in 5 ml of water and the solution was diluted to 100 ml with water. When few drops of this reagent were added to the test filtrate, a brown colour precipitate was formed indicating the presence of alkaloids.

D) Hager's Reagent:

A saturated aqueous solution of picric acid was employed for this test. When the test filtrate was treated with this reagent, yellow precipitate was formed indicating the presence of alkaloids.

6.6.3 Test for Glycoside:

A) Foam test (Saponins)

A few mg of the test residue was taken in a test tube and shaken vigorously with small amount of sodium bicarbonate and water. If stable, characteristic honeycomb like froth was obtained, saponins are present.

B) Killer Killiani:

In 2 ml of sample extract, glacial acetic acid, 1 drop of 5% Fecl3 and conc. H2SO4 was added. Formation of reddish-brown colour at the junction of two liquid layers and upper layer appeared bluish-green, indicates the presence of glycosides.

6.6.4 Test for Tannins and Phenols:

The test residue of each extract was taken separately in water, warmed and filtered. Tests were carried out with the filtrate using following reagent:

A) Ferric chloride test:

A 5 % solution of ferric chloride in 90 % alcohol was prepared. Few drops of this solution was added to a little of the above filtrate. If dark green or deep blue color was obtained, tannins are present.

B) Lead acetate test:

A 10 % w/v solution of basic lead acetate in distilled water was added to the test filtrate. If precipitate is obtained, tannins are present.

C) Potassium dichromate test:

On an addition of a solution of potassium dichromate in test filtrate, dark colour was developed, tannins are present.

D) Bromine water test:

The bromine water was added to the aqueous solution of extract. Development of orange precipitate indicated the presence of tannins.

6.6.5 Test for Flavonoid:

A) Shinoda test:

A small quantity of test residue was dissolved in 5 ml of ethanol (95 % v/v) and treated with few drops of concentrated hydrochloric acid and 0.5 g of magnesium metal. The pink, crimson or magenta colour was developed within a minute or two, if Flavonoids are present.

B) NaOH+ Acid test:

Dilute ammonia solution was added to the aqueous solution of extract solution till discoloration was observed. Few drops of concentrated sulphuric acid was added from the side of test tube. Yellow colour was observed indicate the presence of flavonoids.

6.6.6 Test for Proteins:

A) Biuret test:

A few mg of the residue was taken in water and 1 ml of 4 % copper sulphate was added to it. Violet or pink colour was formed, if proteins are present.

B) Xanthoproteic test:

A little residue was taken with 2 ml of water and 0.5 ml of concentrated nitric acid was added to it. Yellow color was obtained, if proteins are present.

6.6.7 Test for Amino acid:

A) Ninhydrin test:

The ninhydrin reagent is 0.1 % w/v solution of ninhydrin in n-butanol. A little of this reagent was added to the test extract. A violet or purple colour was developed, if amino acids are present.

6.6.8 Test for Carbohydrate:

A) Molisch's test:

This reagent was prepared by dissolving 10 g of alpha- napthol in 100 ml of 95 % alcohol. A few mg of the test extract was placed in a test tube containing 0.5 ml of water, and

it was mixed with two drops of Molisch's reagent. To this solution, was added about 1 ml of concentrated sulphuric acid from the side of the inclined test tube, so that the acid formed a layer beneath the aqueous solution, without mixing with it. If a red brown ring appears at the common surface of the liquids, sugars are present.

B) Barfoed's test:

This reagent was prepared by dissolving 13.3g of crystalline neutral copper acetate in 200 ml of 1 % acetic acid solution. The test residue dissolved in water and heated with a little of the reagent. If a red precipitate of cuprous oxide is formed within two minutes, mono-saccharides are present.

6.7 Physico-chemical Evaluation:

6.7.1 Loss on Drying at 110°C

1. Place the weighed crucible with 5 gm material in tilted lid position in the oven at 105°C for at least 2 hours.
2. Place the crucible in the desiccator by using tongs or gloves and allow cooling for at least 30 minutes.
3. It was taken out and weighed again and again at regular interval till the consistent weight was achieved. The percentage of difference was considered as loss on drying at that particular temperature.

Deterioration time of the plant material depends upon the amount of water present in plant material. If the water content is high, the plant can be easily deteriorated due to fungus.

6.7.2 Refractive Index

Abbe's refractometer is used to measure the refractive index of the given ghrita. Using a particular monochromatic light source, the apparatus is calibrated with water as the liquid. The micrometer screw is adjusted to focus the boundary between the bright and dark regions, then the refractometer scale is adjusted to place the cross wire of the telescope exactly on the boundary between the bright and dark regions. The same process is repeated after the equipment is calibrated. A drop of water was placed on the prism and the drive knob

was adjusted in such a way that the boundary line intersects the separatrix exactly at the centre, and reading was noted.

Distilled water has been found to have a refractive index of 1.3325 at 25˚C. The difference between the reading and 1.3325 gives the error of the instrument. If the reading is less than 1.3325, the error is minus (-) then the correction is plus (+) if the reading is more, the error is plus (+) and the correction is minus (-). Refractive index of Ghrita was determined using 1 drop of the sample. All fats are composed of various types of fatty acids and their derivatives, such as esters in various ratios. With changes of temperature or any other ingredients, the basic constitution of particular fat varies. Such variation may be due to the any impurities added due to any reason. Refractive index varies with temperature and wavelength.

6.7.3 Specific Gravity

Specific gravity bottle was cleaned and shaken with acetone and then with ether. Bottle was dried and weight was noted down. The specific gravity bottle was filled with the ghrita and weight was noted. The procedure was repeated using distilled water in place of Ghrita. The weight of the empty bottle with stopper was recorded. About 10 gms of Ghrita was taken, which was later cooled in a desiccator. The Ghrita was then transferred into the bottle. Then the weight of the bottle and Ghrita was noted. The bottle was again filled completely with 10 ml distilled water. The stopper was placed and the bottle was kept under constant temperature. The bottle was then taken outside, wiped clean, dried and noted. Now determine the weight of the bottle and the contents. The bottle was emptied and thoroughly cleaned, then filled with distilled water and weighed.

The weight of lipid material is affected by the factors such as basic constitution, dissolved constituents used in the preparation of formulation, or any other compound or excipient, which may be used during the process *etc*. In case of fat, it may also change with thermal fluctuations.

6.7.4 Rancidity Test

1ml of melted ghrita was mixed with 1ml of conc. HCl and 1ml of 1% solution of phloroglucinol in diethyl ether and then mixed thoroughly with the fat acid mixture. A pink

color indicates that the fat is slightly oxidized while the red color indicates that the fat is definitely oxidized.

6.7.5 Acid Value

10g of Ghrita was taken in a conical flask. To it was added 50 ml of acid free alcohol-ether mixture (25 + 25ml) previously neutralized by the addition of 1 ml of Phenolphthalein solution and titrated against 0.1N potassium hydroxide solution. End point is figured out with the appearance of pale pink colour which persists for 15 seconds.

The acidity is affected with the process of oxidation as triglycerides get converted in to fatty acids and glycerol. Liberation of fatty acid is the outcome of hydrolysis, thermal effects and lipolytic enzymes as lipase. So, of acid value varies linearly with rancidity. In present work, acid value of Ghrita was found to be below 2 which indicated better quality.

6.7.6 Saponification Value

1. 2g of fat in a tared beaker was weighed and dissolved in about 3ml of the fat solvent (ethanol /ether mixture).
2. The contents of the beaker were quantitatively transferred three times with a further 7ml of the solvent.
3. 25ml of 0.5N alcoholic KOH was added and mixed well, which was attached to a reflux condenser.
4. Another reflux condenser as the blank with all other reagents was present except the fat was set.
5. Both the flasks were placed in a boiling water bath for 30 minutes.
6. The flask was cooled at room temperature.
7. Now added phenolphthalein indicator to both the flasks and titrated with 0.5N HCl.
8. Noted down the endpoint of blank and test.
9. The difference between the blank and test reading gives the number of millilitres of 0.5N KOH required to saponify 1g of fat.

Saponification value or number of fat = mg of KOH consumed by 1g of fat.

The long chain fatty acids present in fats have a low saponification value because they have a relatively fewer number of carboxylic functional groups per unit mass of the fat,

when compared to short chain fatty acids. Medium-chain triglycerides are considered as good biologically inert source of energy that is easy for the human body to metabolize. They passively diffuse from the GI tract to the portal system without requirement for modification like long-chain fatty acids or very-long-chain fatty acids.

6.7.7 Iodine Value

10ml of fat sample was pipetted out and dissolved in chloroform to an iodination flask labelled as “TEST”. 20ml of Iodine Monochloride reagent was added into the flask. The contents in the flask are thoroughly mixed. Then the flask is allowed to stand for half an hour in the dark.

Set up a BLANK in another iodination flask by adding 10ml Chloroform to the flask.

Add to the BLANK, 20ml of Iodine Monochloride reagent and the contents were mixed in the flask thoroughly. The BLANK was incubated in dark for 30 minutes. Meanwhile, the TEST was taken out from incubation after 30 minutes and 10 ml of potassium iodide solution was added into the flask.

The stopper and the sides of the flask was rinsed using 50 ml distilled water. The “TEST” against standardized sodium thiosulphate solution was titrated until a pale straw colour is observed. About 1ml starch indicator was added into the contents in the flask, and a purple colour is observed. The titration is continued until the color of the solution in the flask turns colourless. The disappearance of the blue colour is recorded as the end point of the titration.

Heat increases the dissociation and unsaturation of the molecules of compound. Physicochemical analysis such as loss on drying at 110°C, water soluble extractive, Total ash, Acid insoluble ash, Alcohol soluble extract. Specific gravity and refractive index tests were carried out.

6.8 Quantitative estimation:

Based upon the result obtain from preliminary phytochemical screening quantitative estimation of detected phytocontituents was carried out by standard procedure;

6.8.1 Determination of total phenolic content: (Hagerman et al., 1998)

Phenolic compounds were reported to be active, quenching oxygen-derived free radicals by donating hydrogen atom or an electron to the free radicals (Wanasundara and Shahidi, 1996). Also phenolic compounds of plant materials have been shown to neutralize free radicals in various in-vitro models (Ruch et al 1999).

Principle:

It is based on the principle that Folin Ciocalteau reagent causes the reduction of total phenolic content in the sample forming blue colour which gets detected at 765nm.

Reagents:

- Sodium carbonate: 20%
- Folin Ciocalteau reagent (FCR): 2N
- Triple distilled water (TDW).

Procedure:

Accurately weighed 100 mg of the sample was dissolved in 100 ml of TDW to make the stock solution. This was further diluted 5 times and 1ml of this resultant solution was transferred to a test tube. To this 8 ml of TWD and 0.5 ml of 2N Folin Ciocalteau reagent (FCR) were added to the test tube followed by a vigorous mixing. After 5 minutes, about 1.5 ml of 20% sodium carbonate was added to each of the test tube following proper mixing. Then, finally the whole prepared solution was kept aside for 2 hours, after which the absorbance was recorded at 765 nm against blank using UV spectrometer and the total phenolic contents were expressed in terms of Gallic acid equivalent (mg g^{-1} of dry mass).Standard curve was prepared by using Gallic acid as standard with different concentration i.e. (1-10microgram per ml).

6.8.2 Determination of flavonoid content: (Chang et al., 2002)

Flavonoids comprise a large group of secondary plant metabolites. Presently more than 5000 individual compounds are known, which are based on very few core structures. Their multitude derives mainly from the various hydroxylation patterns (up to six hydroxy groups) and ether substitution by simple methylation or diverse mono- and di-saccharides. Their function in plants themselves most likely involves screening of UV light, in situ radical scavenging, anti-feeding effects (astringency), etc. Flavonols are a major group of

flavonoids, which occur mainly in the form of glycosides in plants. The most common aglycons are quercetin, myricetin and kaempferol.

Principle:

The method is based on the principle that aluminum trichloride forms a flavonoid-aluminum complex having the absorbtivity maximum at 435 nm.

Materials:

- Quercetin (SigmaAldrich)
- Aluminum chloride Hexahydrate (SDFCL, Mumbai),
- 95 % alcohol (Oasis, Mumbai),
- Sodium acetate (Rankem, Mumbai)

Procedure:

The total Flavonoid content was determined as, 0.1 ml of 10 % aluminum trichloride hexahydrate ($AlCl_3$ $6H_2O$) in methanol was mixed with about 0.5 ml test solution,1.5ml 95 % alcohol and 0.1 ml 1 M sodium acetate. Make up to 5 ml with water and incubate the resultant mixture at $20^{\circ}C$ for 40 min. Absorption readings at 435 nm were taken against a blank sample. The total Flavonoid content was determined using a standard curve with Quercetin (2-10 µg/ml) as the standard. The mean of three readings was used and expressed as mg Quercetin of equivalents (QE)/100 g of extract.

6.8.3 Determination of total saponin content: (Desai et al., 2011)

Materials:

- 80% Methanol,
- 8% Vanillin,
- Ethanol,
- 72% H_2SO_4,
- Diosgenin

Procedure:

The Saponin quantification was carried out in accordance with Helaly method with slight modification. The extract was dissolved in 0.5 mL Methanol 80%. The following were

added to the solution: around 0.5 mL of 8% vanillin in ethanol and 5 ml of 72% H_2SO_4 in water. The mixing of the reagents was carried out in a thermostat ice bath at 0º C. The mixture was then set in a thermostat at 60º C for 20 min and at 0º C for 5 min and then measured at a wavelength of 544 nm. A calibration curve was constructed using purified Saponin as standard Saponin which was also treated in a similar manner.

6.8.4 Estimation of tannin: (Sadashivam and Manickam., 1996)

Vanillin hydrochloride method:

Principle – The vanillin reagent will react with any phenol that has an unsubstituted resorcinol/ phloroglucinol nucleus and forms a coloured substituted product which is measured at 500nm.

Materials:

Vanillin HCl reagent – mix equal volume of 8%HCl in methanol and 4% vanillin in methanol. The solution was mixed just before use and avoids using even if it is slightly coloured.

Catechin - 1mg/ ml of methanol.

Working standard – the above stock solution was diluted 10 times. 10 ml to 100ml
(100 µg/ml).
Preparation of extract – 1g of sample was ground in 50ml of methanol, with occasionally mixing by swirling. After 20-25 hours it was centrifuged and the supernatant was collected.

PROCEDURE:

- Around 1 ml of the supernatant was pipette out.
- About 5ml of vanillin HCl reagent was added quickly.
- The reading was taken in spectrophotometer at 500nm after 20 min.
- The blank was prepared with vanillin HCl reagent alone.
- The graph was prepared of standard with 20- 100µgCatechinusing the diluted stock solution.

6.8.5 Estimation of alkaloid content: (Wagner and Bladt, 1996)

The estimation of the total alkaloid content was done as per the gravimetric method. 5 gm powdered drug is extracted repeatedly using 0.1N $H2SO_4$ in an ultrasonic bath. The solution is filtered; the mixed acid solution is washed with 4 successive quantities of 25 ml chloroform. The chloroform washing rejected, acid solution is basified with dilute ammonia solution and extracted with diethyl ether. The combined diethyl ether extracts are washed with 5 ml distilled water and ether is evaporated to dryness in a weighed beaker on a water bath. Residue is dried to constant weight at 105^0C.

6.8.6 Estimation of total carbohydrates by phenol sulfuric acid method: (Sadashivam and Manickam., 1996)

Principle:

In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This forms a green colored product with phenol and absorption maximum at 490 nm.

Materials:

- **Phenol 5 %:** Redistilled (reagent grade) phenol (50 g) dissolved in water and diluted to one liter.
- **Sulfuric acid 96 %:** (reagent grade).
- **Standard glucose: Stock solution-** 100 mg in 100 ml of water.
- **Working standard**: 10 ml of stock solution diluted to 100 ml with distilled water.

Procedure:

Plant material (100 mg) was hydrolyzed with 5 ml of 2.5 N HCl for three hours on water bath and cooled at room temperature and filtered. The solution was neutralized with sodium carbonate until the effervescence ceased and the volume was made up to 100 ml with water. The solution was centrifuged and supernatant was collected. Of the working standard 0.2, 0.4, 0.6, 0.8 and 1 ml was pipette out in series of test tubes. Similarly of the sample solution 0.1 and 0.2 ml was pipette out in two separate test tubes. The volume was made up to 1 ml with water and 1ml of water was set as a blank. Phenol solution (1 ml) and 96 % sulfuric acid (5 ml) was added to each test tube. After 10 min the contents in the test tubes were shaken and placed in water bath at 25-30 ^{0}C for 20 min. and color was read at 490 nm.

The amount of carbohydrates was calculated as a Dextrose equivalent from the calibration curve of Dextrose standard solutions, and expressed as mg of Dextrose / g of plant material.

6.8.7 Determination of total flavonol: (Kumaran and Karunakaran, 2006).

Accurately measured 1 mL of plant extract (10 mg/mL) was mixed with 1 mL aluminium trichloride (20 mg/mL) and 3 mL sodium acetate (50 mg/mL). The absorbance at 440 nm was read after 2.5 hr. The absorbance of standard rutin solution (0.5 mg/mL) in methanol was measured under the same conditions. (Kumaran and Karunakaran, 2006).

6.9 Chromatographic evaluation:

6.9.1 Thin layer chromatography (TLC): (Wagner et al,. 1996)

The extracts were subjected to thin layer chromatographic studies, to find out the probable number of compounds present in them. The details of the procedure are as follows:

i) Preparation of the plates:

The adsorbent/stationary phase used for thin layer chromatography was silica gel G. About 25 g of silica gel G was taken in a glass mortar and about sufficient water was added to it. The mixture was stirred with glass rod until it became homogeneous and allowed to swell for 15 minutes and then additional water was added to it with stirring. This suspension was then uniformly spreaded immediately on plates.

ii) Drying and storage of plates:

The freshly coated plates were then air dried and stacked in a drying rack and were heated in an oven for 30 minutes at 110 °C. Activated plates were kept in a desiccator, till required for further use.

iii) Sample and Standard preparation:

Sample and Standard stock solution where prepared in methanol.

iv) Application of the sample:

The test samples were applied in the form of a spot, with the help of fine capillaries.

v) Standard: Gallic acid (Sheetalet al., 2007)

vi) Developing solvent system:

Toluene: Ethyl Acetate: Methanol: Formic Acid (6:3:1:0.5) (Sheetal et al., 2007)

vii) Development of TLC plates:

Chromatographic rectangular glass chamber was used in the experiments. To avoid in sufficient chamber saturation. Different mobile phase were tried but the satisfactory resolution was obatained in the solvent system mentioned in figure . After development of plates, they were air-dried and number of bands wee noted and R_f values were calculated.

The R_f value (Retention Factor) was calculated as follows:

R_f = Distance traveled by the sample/ Distance traveled by the s

viii) Comparison with standard:

The extracts of Inhouse and Marketed formulations were compared with Berberine. Taking berberine as a standard. This was done by the following method;

ix) Standard TLC method:

In this method TLC of arjuna ghrita sample were carried out by using the solvent system Toluene: Ethyl Acetate: Methanol: Formic Acid (6:3:1:0.5) in which maximum number of spots were obtained and then the comparision were carried out using gallic acid as a standard. Results were obtained by calculating R_f values of both the inhouse and marketed formulations and then comparing them with Rf value of standard.

6.9.2 High Performance Liquid Chromatography (HPLC)

Ultra filtration liquid chromatography (RP-UFLC)(UFLC Shimadzu, SPD-M20A with PDA Detector)

The sample was standardized using Gallic Acid as a marker compound after confirming its presence through thin layer chromatography. The chromatographic studies were performed on C_{18} analytical column (Spinchotech Pvt. Ltd. Enable). Mobile phase were prepared in closed solvents bottles and sonicated for about 20 min. Gradient mobile phase containing **Warer:Acetonitrile (80:20% v/v)**for gallic acid were used that gave the best resolution of peak at less retention time for gallic acid with flow rate of 1.0 ml/min. The maximum absorption wavelength of gallic acid was found to be 254 nm, 358 nm hence

selected as detection wavelength for analysis (Heena A.R. et al., 2018) (Sahani and Jain et al., 2018).

i) Preparation of standard solution:

10 mg of standard samples were weighed and dissolved in 5 ml methanol by means of sonication for 15 min. The solution was diluted up to 10 ml with methanol (1 mg/ml). Pipette out 1 ml solution from stock solution and diluted up to 10 ml with methanol (100µg/ml) and filtered through 0.2 µ nylon membrane filter and used for HPLC analysis.

ii) Preparation of sample solution:

10 mg of inhouse and marketed sample were weighed and dissolved in 5 ml methanol by means of sonication for 15 min. The solution was diluted up to 10 ml with methanol (1 mg/ml). Pipette out 1ml solution from stock solution and diluted up to 10 ml with methanol (100 µg/ml) and filtered through 0.2 µ nylon membrane filter and used for HPLC analysis

iii) Procedure:

The chromatographic conditions were set as per the given parameters and mobile phase was allowed to equilibrate with stationary phase as was indicated by the steady baseline. The standard solution and prepared samples was injected using Hamilton syringe and the chromatograms were recorded for the standard sample.

Table 5: Chromatographic condition for HPLC

Sr No	Parametrs	Remarks
1	HPLC	UFLC Shimadzu
2	Standard	Gallic Acid
3	Column	C18
4	Mobile phase	Water: Acetonitrile (80:20% v/v)
5	Detection Wavelength	254 nm and 238 nm
6	Injection volume	20 µl
7	Flow rate	1.0 ml/min
8	Temperature	25±2° C

6.10 Preclinical Evaluation:

i) Preparation of dose:

Aqueous solution of each sample was prepared in 0.5% CMC solution and two different doses were decided (100 mg/kg, 200 mg/kg b. w.) for the activity. The samples were given by oral route with the help of oral gavage.

ii) Animal Studies:

Swiss Albino female Mice weighing 20-30g were obtained from the Animal House (Reg. No 92/1999/CPCSEA Dated - 28/04/1999) (Reg.No. IAEC/UDPS/2018/47.), Department of Pharmaceutical Sciences, RTMNU, Nagpur and were kept under standard lab condition. The animals were allowed to acclimatize to the environment for 7 days before the commencement of experiments. Swiss Albino female mice were maintained on 12-h light, 12-h dark cycles and given food and water ad libitum. The experimental protocol was approved by Central Animal Ethical Committee of RTMNU Nagpur University (dated 04/08/2018). Grouping of animals was based upon individual studies and the extracts were given orally.

iii) Study Design:

The study consisted of 21 female Swis Albino mice equally divided into seven groups containing 3 mice in each designated as group 1 (served as normal group), group 2 (served as control group), group 3 (served as standard (STD) group), group 4 (served as a inhuose low dose (LD) group) and group 5 (served as inhouse high dose (HD) groups), group 6 (served as marketed low dose) and group 7 (served as a marketed high dose). The body weight of animals was measured at the intervals of 7 days for 63 days during the study.

iv) Induction:

The high fat diet included pallets prepared by combination of diet wheat four 53%, milk powder 25.5%, cholesterol 1.5% and vanaspati ghee 20%, were mixed well. Thereafter, water was added to the above mixture. From this dough, the pellets of high fat diet were prepared. These pellets were baked at 100ºC for two hours and stored in air tight container

the pellets were administered to the rats as a regular diet for a period of 42 days (Pandey et al., 2010).

v) Experimental protocol:

- Grouping of animals in normal and high fat diet was done.
- Animals were given the feed according to the group for 42 days.
- After each week on 7th day, animals were weighed and the weight was recorded.
- After the completion of 42 days of induction, animal's blood was withdrawn by Retro Orbital Plexus method and lipid profile was carried out.
- After the completion of blood profile estimation the treatment was carried out for 21 days where standard group animals were treated with standard drug daily (dose 50 mg/kg).
- The normal and control group were not subjected to treatment.
- The In-house and marketed sample treatment was given in 2 doses i.e. in high dose and low dose respectively.
- On 22nd day of end of treatment the animal's blood was withdrawn by Retro Orbitol Plexus method and Lipid profile was carried out, which include TC, TG, HDL, VLDL, and LDL.

vi) Treatment schedule:

The HFD standard group of mice was treated with fenofibrate at a dose of 50 mg/kg. Two different doses i.e 100 mg/kg (Low dose) sample was given to inhouse low dose and marketed low dose group. And 200 mg/kg (High dose) to inhouse high dose and marketed high dose group in 0.5% CMC particularly once a day for 21 days.

Table 6: Grouping of animals

Sr no.	Groups	No. of Animals
1.	Normal	6
2.	High fat diet (HFD)	6
3.	HFD + Standard (Fenofibrate)	6
4.	HFD + Low dose inhouse formulation (100 mg/kg)	6
5.	HFD + High dose inhouse formulation (200 mg/kg)	6
6.	HFD + Low dose marketed formulation (100 mg/kg)	6
7.	HFD + High dose marketed formulation (200mg/kg)	6

vii) Biochemical Evaluations:

The blood was withdrawn by Retro Orbital Plexus method of mice on the 0th day and 21st day of treatment. The serum from the blood was separated by centrifugation method at 3000 rpm and was subjected to evaluation of Lipid Profile which includes Cholesterol, TG, HDL, VLDL, and LDL. The lipid profile evaluation was carried out by

viii) Histopathological studies:

The sample of liver and kidney were collected immediately after sacrificing mice and fixing sample in 10 % neutral buffered formalin overnight. The sample of liver and kidney were dehydrated in a graded series of ethanol, cleared with xylene, embedded in paraffin and serially sectioned at 4 µm. Slices were placed on glass microscope slides in traceable order, stained with hematoxylin and eosin and finally analysed under light microscopy (Tessaro et al., 2015).

ix) Biochemical estimations:

a) Estimation of proteins and DNA:

Preparation of homogenate for estimation of protein content:

For estimation of protein, tissue was first homogenized in 5% trichloro acetic acid and then centrifuged. The pellets were then washed with 10% trichloro acetic acid and then again suspended in 5% trichloro acetic acid. It was then kept for 15 min in a water bath maintained

at 90°C. The contents were centrifuged and the precipitated proteins were suspended in 0.1 M Tris–HCl, pH 7.4, and the protein content was estimated by the method of Lowry et al. (1951) (Schneider, 1957).

b) Estimation of protein content: (Lowry et al., 1951):

- **Principle:** It is based on the principle that copper forms a complex with protein (tyrosine major and tryptophan to minor extent) and causes reduction of Folin ciocalteau reagent (phosphotungstic and phosphomolybdic acid) forming blue colour having absorptivity maximum at 600nm.
- **Reagents:**

Alkaline copper reagent

Copper sulphate: 20mg/L

Sodium potassium tartrate: 20mg/L

Sodium carbonate: 20g/L

Sodium hydroxide: 40g/L

Folin Ciocalteau reagent (FCR): 2N

- **Procedure:**

To 0.5ml of above homogenate, 6.0ml of alkaline copper reagent was added. This was then mixed properly and allowed to stand for 10 minutes. To this 0.5ml of Folin ciocalteau reagent was added with proper mixing and allowed to stand for 30 minutes. Finally, absorbance was taken at 600nm.

Standard curve was prepared by using standard bovine serum albumin in the concentration range of 10, 20, 40, 80 and 160 microgram/ml.

Estimation of DNA content: (Burton method, 1956):

Principle: It is based on the principle that diphenylamine forms a complex with deoxyribose moiety of DNA having absorptivity maximum at 600nm.

Reagents:

Perchloric acid: 0.5M

Burton reagent

Diphenylamine: 15g/L

Sulphuric acid: 0.25M

Acetaldehyde: 0.05ml/L

Procedure:

To the tissue sample, enough perchloric acid was added to cover the sample. It was then heated in a water bath for about 90 minutes at temperature between 70 -800C. This was then centrifuged at 300g to remove the cellular debris. To 1ml of the above supernatant, 2ml of Burton reagent was added, mixed properly and allowed to stand for 18 hours at 300C. Finally, absorbance was taken at 600nm. Standard curve was prepared following the same procedure by using standard DNA sample in the concentration range of 40, 80, 120, 160 and 200 microgram/ml.

Estimation of Catalase: (Chance and Maehly 1955):

Principle: it is based on the principle that hydrogen peroxide in phosphate buffer (pH 7.0) reacts with the diluted sample causing release of the Catalase enzyme which gets absorbs at 240nm.

Reagents:

Phosphate buffer

Hydrogen peroxide: 6%

Procedure: To 1ml of supernatant, 2.25ml of phosphate buffer was added followed by the addition of 0.65ml of H2O2. The absorbance was then taken at 240nm and compared with 6% H2O2. First reading was taken after 3 minutes and final until reading was not constant.

Estimation of lipid peroxidation (LPO):

Principle: It is based on the principle where MDA (Malonyl dialdehyde) forms a complex with TBA in presence of sodium dodecyl sulphate (SDS) and n-butanol-pyridine mixture which gets absorbed at 532nm.

Reagents:

Sodium dodecyl sulphate (SDS)

20 % acetic acid solution (pH 3.5)

Thiobarbituric acid

n-butanol and pyridine (15:1, v/v)

Procedure:

To 0.2 mL of tissue homogenate, 0.1 mL of 8.1 % SDS, 0.75 mL of 20 % acetic acid solution (pH 3.5) and 0.75 mL of 0.8 % aqueous solution of TBA was added in stoppered tubes. The mixture was made up to 2 mL with distilled water, and then heated in an oil bath at 95 °C for 60 min. After cooling with tap water, 0.5 mL of distilled water and 2.5 mL of mixture of n-butanol and

Pyridine (15:1, v/v) was added and shaken vigorously. After centrifugation at 3000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured against blank containing 0.2 mL of distilled water in place of sample (Nehius and Samuelson, 1968).

Statistical analysis:

The experimental results are expressed as mean ± SEM, with six animals in each group followed by one-way and two-way analysis of variance (ANOVA). Newman-keuls test and Bonferroni-post test for multiple comparisons were applied for determining the statistical significance between different groups. Graph Pad Prism, version 5 software, was used for all statistical analysis. P values <0.05 were considered to be significant.

RESULT…….

7. RESULTS:

7.1 Physicochemical Evaluation:

Table No 7: Physicochemical Evaluation

Sr No	Parameter	Result	
		Marketed Sample	In-house Sample
1	Organoleptic ▪ Color ▪ Odour ▪ Taste ▪ State ▪ Texture	▪ Yellow ▪ Ghee like ▪ Characteristic ▪ Semi-solid ▪ Smooth	▪ Yellow ▪ Ghee like ▪ Characteristic ▪ Semi-solid ▪ Smooth
2	Ph	3.2	3.2
3	Specific gravity	0.9426	0.9394
4	Acid value	0.9831	1.5147
5	Saponification value	132.82	124.82
6	Ester value	123.3035	123.3035
7	Fat Content	76.58%	76.50%

Table no 8: Physicochemical Parameter of Arjuna bark

Sr No	Parameters	Result	
		Marketed	**In-house**
1	Moisture Content	11.5	10.19
2	Total Ash	17.25	17.49
3	Alcohol soluble extractive	23.36	28.48
4	Water soluble Extractive	20.21	20.50

7.2 Preliminary Phytochemical Screening:

Table No 9: Phytochemical Evaluation

Sr No	Phytoconstituents	Test	Result	
			Marketed Sample	In-house sample
2	Carbohydrate	Molisch's test	+	+
3	Flavonoid	Lead Acetate test	+	+
4	Proteins	Ninhydrin test	+	+
5	Glycoside	Keller-Killani test	+	++
		Saponin Glycoside	+	+
6	Triterpenoid	Liebermann-Burchard's test	+	+
7	Lactones	Legal's test	+	+
8	Phytostrerols	Salkowski reaction	++	++
9	Phenolic compound and tannins	5% $Fecl_3$ Test	++	++

7.3 Quantitative Estimations:

Table No 10: Quantitative Estimation

Sr No	Parameters	Result	
		Marketed(mg/gm)	**In-house(mg/gm)**
1	Total Phenol Content (mg/g) Eq.Gallic acid	435.98	499.8
2	Total flavonoid content (mg/g) Eq. Quercetin	204.91	209.91
3	Total Saponin content (mg/dl) Eq. Diosgenin	-	-
4	Tannin Content(mg/g) Eq. Tannic acid	9.5643	9.95568
5	Total alkaloids (% w/w)	-	-

Eq: Equivalent Values are expressed as Mean ± SEM (n=3)

7.4 Chromatographic Evaluations:

7.4.1 Thin Layer Chromatography:

Rf value = Distance travelled by solute /Distance travelled by solvent front

Table No 10: Chromatographic Evaluation

Sr.No.	Samples	Solvent System	No. of Spot	Retention Factor
1.	Standard Gallic Acid	Toluene: Ethyl Acetate: Methanol: Formic Acid (6:3:1:0.5) (ml)	1	0.35
2.	Marketed	Toluene: Ethyl Acetate: Methanol: Formic Acid (6:3:1:0.5) (ml)	3	0.35,0.39,0.45
3.	In-house	Toluene: Ethyl Acetate: Methanol: Formic Acid (6:3:1:0.5) (ml)	5	0.39,0.32, 0.35,0.41, 0.35

SUMMARY & DISCUSSION…….

8. SUMMARY AND DISCUSSION:

The present research work deals with the physicochemical, phytochemical and preclinical evaluations of arjuna ghrita formulation as cardio protective. The ghrita formulation includes the combination of arjuna bark extract and go-ghrita (Simple ghee). Various pharmaceutical parameters for Ghrita Formulations were performed which includes physicochemical, phytochemical, chromatographic evaluation. The chemical nature of the active constituents present in the formulation can be identified by performing preliminary phytochemical screening of that formulation. The phytochemical screening of ghrita formulation showed the presence of flavonoids, tannins, saponin, alkaloids, phenol and carbohydtrates in both the formulations.

The quantitative estimation performed in the present study depicted the presence of polyphenol, flavonoid, saponin, tannin, alkaloidal, carbohydrate and flavonols content in both inhouse and marketed formulations.

With the help of TLC one can easily determine purity and quantity of sample, examination of reaction, identification of compound in a mixture, separation of multicomponents in pharmaceutical formulations. TLC was done for the determination of different components present in the formulations. In TLC R_f value of one of the constituent matches with the standard Gallic Acid in Toluene: Methanol: Formic Acid: Ethyl Acetate (6:1:3:0.5) as a solvent system in both the formulations.

The quantitative estimation also depict the presence of various polyphenol, tannins, flavonoids in higher amount whereas the other components in considerable quantity.

CONCLUSION…….

9. CONCLUSION:

- In the present research work, attempts were made for the physicochemical, phytochemical and chromatographic evaluation of Arjuna ghrita for its cardio protective effect.
- The physicochemical parameters were evaluated and were found to be within the prescribed limits as per standard values mentioned in API.
- Pharmaceutical parameters of ghrita formulations were evaluated and found to be within the prescribed limit as per standard values of IP.
- Phytochemical study revealed the presence of mainly alkaloids, flavonoids, tannins, polyphenol, saponin which were found to be in considerable amount in ghrita preparations.
- The quantitative estimation performed of formulation reveals the presence of polyphrnols,flavonoids, tannins were found in higher amount whereas the other were found in considerable amount.
- The TLC analysis of ghrita formulation using the solvent system Toluene: Ethyl Acetate: Methanol: Formic Acid in a ratio 6:3:1:0.5 showed the presence of gallic acid in both the inhouse and market sample with the Rf value comprising with std gallic acid value.
- In conclusion, we have successfully evaluated the physicochemical, phytochemical evaluations of ghrita formulation which will act as a referential source for standardization of other formulations.
- The present research work suggest that, there are various synthetic drug present for treatment of CVD which can produce major side effects but the use of this type of ancient ayurvedic ghrita formulations in combination is efficacious and safe in the treatment of CVD.
- The study also shows some better results when compared with the marketed formulation, one can conclude that using the physiologically shaded bark in ancient ghrita formulation can prevent CVD and can also provide the area of research for the future scientist.
- On considering all the evaluated parameters along with the research done, it can also be concluded that as ghrita is already having various lipid lowering capacity hence the combination of medicated ghrita and physiologically shaded arjuna bark can potentiate the effect.

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