3.1. Ashvagandharishta (Ayurvedic Pharmacopoeia of India 2008, Prabhakar Rao et al., 2008)

As stated earlier, Ashvagandharishta is a fermented liquid preparation containing 5-10% of self-generated alcohol. The yeast cultures isolated form the *W. fruticosa* flowers have been described in the earlier chapter. This chapter is dedicated to the detailed discussion of the process of perpetration of Ashvagandharishta using these yeasts. The methodology followed was strictly as described in Ayurvedic Pharmacopeia of India. All the ingredients were exactly in their prescribed proportions to make the pre-fermentation *Kwath*, which was divided in equal parts. In one, *W. fruticosa* flowers was added and another was inoculated by the yeast consortium. These two formulations were compared with several physicochemical and analytical parameters, which are presented in this chapter.


Asvagandharishta composition as per Bhaishjya Ratnavali (Chaudhary et al., 2006)
Ashvagandharishta composition as per Ayurvedic Pharmacopoeia of India.


### 3.1.2. Preparation protocol as per Ayurvedic Pharmacopoeia of India

Following is the most accepted protocol for Arishta preparation. Some variations in the protocol are also practiced but the main theme is more or less the same. Authenticated herbal raw materials of pharmacopoeial quality should be taken. Clean, dry and powder *Kvatha dravyas* individually and pass through the sieve number 44 to obtain coarse powder. Clean, dry and powder *Prakshepa dravya* of the formulation composition individually and pass through the sieve number 85 to obtain finer powder. Add specified amounts of water to the *Kvatha dravya*, soak overnight, heat and bring it boil to reduce to one eighth of the original volume. Cool and filter through muslin cloth to obtain *Kvatha*. Transfer the filtrate to a clean container; add specified amount of jaggery or honey and *Woodfordia fruticosa* flowers of the formulation composition. Finally, add the powdered *Prakshepa dravyas* and seal the mouth of the container. Shift the container to the
fermentation room or to an isolated corner away from direct sun. Frequently check for the signs of completion of fermentation process. Filter the fermented material through a clean muslin cloth. Pack in air tight containers and allow maturing (Ayurvedic Pharmacopoeia of India, 2008).

3.1.3. Storage of Asava-Arishta

Prepared Asava-Arishta formulations to be stored in a cool place in tightly closed amber coloured bottle, protect from light and moisture. For large volume storage, wooden casks are preferred however, use of stainless steel tanks are also common at manufacturing units.

3.1.4. Therapeutic uses

Pharmaceutical value of Ashvagandharishta is reviewed in details in the next chapter, while pharmacological significance of the constituent drugs is given in this chapter.

According to the Ayurvedic Pharmacopoeia of India, Ashvagandharishta is prescribed for following conditions.

Murccha (syncope), Apasmara (epilepsy), Sosa (cachexia), Unmada (mania/psychosis), Karsya (emaciation), Arsa (piles), Agnimandya (digestive impairment), Vataroga (neurological disorders).

3.1.5. Human dose (Ayurvedic Pharmacopoeia of India, 2008)

About 15–30 ml orally with equal amount of water after meals twice a day
3.1.6. Ashvagandharishta: Physico-chemical parameters (Ayurvedic Pharmacopoeia of India, 2008)

*Table 3.1.* Physico-chemical parameters of Ashvagandharishta as per Ayurvedic Pharmacopoeia of India.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Physico-chemical parameters</th>
<th>Standard values of Ashvagandharishta</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Appearance</td>
<td>Clear brown liquid without frothing and significant sedimentation with aromatic odour and astringent taste.</td>
</tr>
<tr>
<td>2.</td>
<td>pH</td>
<td>3.50 to 4.50,</td>
</tr>
<tr>
<td>3.</td>
<td>Specific gravity (at 25°C)</td>
<td>1.05 to 1.20</td>
</tr>
<tr>
<td>4.</td>
<td>Total solids</td>
<td>Not less than 18.5 per cent w/v,</td>
</tr>
<tr>
<td>5.</td>
<td>Alcohol content</td>
<td>5-10 percent v/v,</td>
</tr>
<tr>
<td>6.</td>
<td>Presence of Methanol</td>
<td>Absent</td>
</tr>
<tr>
<td>7.</td>
<td>Reducing sugars</td>
<td>Not less than 13 per cent w/v,</td>
</tr>
<tr>
<td>8.</td>
<td>Non-reducing sugar</td>
<td>Not more than 0.70 per cent w/v,</td>
</tr>
<tr>
<td>9.</td>
<td>Total Phenolic content</td>
<td>0.104 to 0.260 per cent w/v, equivalent to Tannic acid</td>
</tr>
</tbody>
</table>
3.2. Review on Pharmacological significance of the constituent drugs used in Ashvagandharishta

Ashvagandharishta is composed of around 28 different herbs and honey or jaggery as sugar or a media for fermentation. These herbs are contributing for medicinal potential of Ashvagandharishta. At individual level these herbs represents diverse biological activities. From Phytochemistry point of view, Ashvagandharishta is an association of several different phytocompounds from different herbs, which thought to work in synergy and uniquely contributes to immunomodulatory, anti-inflammatory, neuroprotective and antioxidant activity. Important individual herbs are summarized for its Ayurvedic importance, chemical composition and pharmacological value.

3.2.1. Ashvagandha (Nadkarni et al., 1976, Umadevi et al., 2012)

Ashvagandha or Indian winter cherry is considered one of the most important herbs in Ayurvedic system of medicine. Ashvagandha is widely recognised for its ability to boost the immune system and help to ward off illness and disease.
Botanical name: *Withania somnifera* (L.) Dunal.

Classification:

- **Kingdom**: Plantae
- **Order**: Solanales
- **Family**: Solanaceae
- **Genus**: Withania
- **Species**: fruticosa

Vernacular names: Ashvagandha, Indian ginseng or Winter cherry, Ashwagandha.

Useful part: Roots and leaves

Ayurvedic Properties

- **Rasa**: Tikta
- **Guna**: Laghu
- **Veerya**: Usna
- **Vipaka**: Madhura
- **Dosha**: Pacifies Kapha and Vata doshas

Chemical composition:

The whole plant as well as specific parts (roots, stems, leaves) of plant extract and its active constituents have been used for the treatment of large number of human ailments. The main constituents of ashwagandha are alkaloids and steroidal lactones (Mirjalili *et al*., 2009). Withanine, somniferine, somnine, somniferinine, withananine, pseudo-withanine tropane, pseudo-tropine, choline, anaferine, anahydrine, isopelletierine are chemical constituents present in it (Kaur *et al*., 2001). The leaves contain a group of steroidal lactone, which are commonly called "Withanolides". Withaferine A has been receiving good deal of attention because of its antibiotic and anti-tumor activity. The
paste prepared out of its leaves is used for curing inflammation of tubercular glands. Fruits and seeds are diuretic in nature.

**Pharmacological values** (Mishra et al., 2000, Varma et al., 2011)

Since Vedic times, Ashvagandha is regarded as wonder herb for its pharmacological value, as an antibiotic, adaptogen, abortifacent, aphrodisiac, anti-inflammatory, sedative and as a nerve tonic. Ashvagandha also has been found to be potent antioxidant, stimulate immune system cells such as lymphocytes and phagocytes. Also counteract the effects of stress and generally promote wellness.

**Important formulations of Ashvagandha** (Ayurvedic Pharmacopoeia of India, 2008)

Ashvagandha is extensively used in Ayurvedic formulations such as Ashvagandha powder, Ashvagandha extract, Ashvagandha ghruta, Ashvagandharishta, Ashvagandha rasayana etc.

**Ashvagandha dosage** (Monograph of *Withania somnifera*, 2004)

The most popular method is to take the Ashwagandha churna (root powder) 3 to 6 g or 300-500 mg of an extract standardized to contain 1.5% withanolides. Ashwagandha extract in tablet form (2-4 tabs. of 250 mg daily).

**Ashwagandha side effects** (Kushwaha et al., 2012)

Excessive doses of ashwagandha have been reported to cause abortions, so pregnant women should avoid this herb. Do not take this herb with other sedatives or anti-anxiety drugs. Large doses can cause diarrhea, stomach upset and vomiting.

**3.2.2. Safed musali** (Desale et al., 2013, Singh et al., 2012)
Safed musali is native to parts of Africa and India one of several species known for its pharmacological activity. It has historical uses in Ayurveda

(http://www.naturalaphrodisiacs.net/herbs and plants/safed-musli-chlorophytum-borivilianum)

**Botanical name:**  *Chlorophytum tuberosum* (Roxb.) Baker

**Classification:**

- **Kingdom:** Plantae
- **Order:** Asparagales
- **Family:** Asparagaceae
- **Genus:** *Chlorophytum*
- **Species:** *tuberosum*

**Useful part:** Roots, leaves

**Vernacular names:** Safed musali, Kuli, Musali, Dravanti, Kucchela

**Ayurvedic Properties**

- **Rasa:** Madhura
Guna: Guru, Snigdha  
Veerya: Sita  
Vipaka: Madhura  
Dosha: It alleviates vata and pitta doshas and aggravates the kapha doshas

Chemical composition (Deore et al., 2010)

Safed Musli contains carbohydrates (35-45%), fiber (25-35%), alkaloids (15-25%), saponins (2-20%), and proteins (5-10%). It is a rich source of over 25 alkaloids, vitamins, proteins, carbohydrates, steroids, saponins, potassium, phenol, resins, mucilage, and polysaccharides and also contains high quantity of simple sugars, mainly sucrose, glucose, fructose, galactose, mannose and xylose.

Pharmacological values

Roots and leaves are used in the treatment of diabetes and known to boosts the general immune system. It is increasingly being used as a health tonic (Khanam et al., 2013). Safed musali traditionally used for its aphrodisiac activity, in male impotency or in oligospermia. It is also widely used as a general health promotive agent and for delaying ageing process. Some of the proven pharmacological activities are immunomodulatory, anthelmintic, antiulcer, anti-stress, anti-cancer, anti-microbial and also have larvicidal activity (Singh et al., 2012).

Important formulations of Musali

Safed musali power, Safed musali tablets, Safed musali capsules and also used in common.

Dosage (Singh et al., 2012)
The appropriate dose of Safed musali depends on several factors such as the user’s age, health, and digestive conditions.

3.2.3. Manjishta (Lavekar et al., 2008)

*Rubia cordifolia* L. is a climbing or scrambling herb, with red rhizomatous base and roots. It is an essential raw drug for the traditional herbal formulations such as Ashvagandharishta, Jaatyadi ghrita, Madhookasavam, Majishthaadi taila, Useerasavam etc.

(bibliotheque.bordeaux.fr and envis.frlht.org)

**Botanical name:** *Rubia cordifolia* (L.)

**Classification:**

- **Kingdom:** Plantae
- **Order:** Gentianales
- **Family:** Rubiaceae
- **Genus:** *Rubia*
Species  *cordifolia*

Useful part: Roots.

Vernacular names: Manjishta, Mantha, Tamaralli, Manditti

Ayurvedic Properties

**Rasa:** Tikta, Kashaya

**Guna:** Guru, Ruksha

**Veerya:** Usna

**Vipaka:** Katu

**Dosha:** Kapha-Pitta Shamaka


Characteristic chemical constituents of the rootstock of this plant are anthraquinone and cyclic peptides. Main anthraquinone constituents are munjistin, purpurin, and pseudopurpurin. Newly reported anthraquinones namely 1-hydroxy-2, 7-dimethylantraquinone, 2-hydroxy-6-methylantraquinone, 2,6-dihydroxyanthraquinone, 1-hydroxy-2-methylantraquinone, nordamnacanthal, physcion, 1,4-dihydroxy-6-methylanthraquinone, 1,4-dihydroxy-2-methylantraquinone, 1,5-dihydroxy-2-thylanthraquinone, 3-prenylmethoxy 1,4-naphthoquinone, 1-hydroxy-2-methoxy anthraquinone, 1,4-dihydroxy 2-methyl-5-methoxy anthraquinone or 1,4-dihydroxy 2-methyl-8-methoxy anthraquinone, 1,3-dimethoxy 2-carboxy anthraquinone and rubiadin have been isolated from *Rubia cordifolia* roots. Three new anthracene derivatives, rubiasins A–C, were isolated from the combined roots and stems of *Rubia cordifolia*

*Pharmacological values* (Bhat *et al.*, 2013)

Powdered dried roots and fruits are taken internally for the treatment of skin diseases and disorders of spleen. It is used for the treatment of major burns, ulcers and bone fractures.
It is considered tonic, antitussive, and useful in chronic low fevers. The roots are used internally in the treatment of abnormal uterine bleeding, internal and external hemorrhage, bronchitis, rheumatism, kidney stones, bladder and gall stones, dysentery etc. The plant is used in the treatment of blood disorders. The roots are anodyne, antiphlogistic, astringent, diuretic, expectorant, styptic and vulnerary.

**Important formulations of Manjishta** (Sharma et al., 2012)

Manjishta is found in many traditional Ayurvedic formulations such as Arishta, Chandanasava, Jatyadi ghrut, Kvatha Curna, Manjishtadi taila, Phala Ghrit and Pinda taila.

**Dosage**

About 1-3 g of powder, 56-112 ml decoction one to three times a day or as directed.

### 3.2.4. Haritaki (The Ayurvedic Pharmacopoeia of India, 2002)

The chebulic myrobalan is of great importance in Ayurvedic medicine. It is known as haritaki in Sanskrit because it is sacred to Shiva (hara). It is an important ingredient of Triphala, a rasayana, which also contains *Terminalia belerica* and *Phyllanthus emblica*. 
Botanical name: *Terminalia chebula* Retz.

Classification:

- **Kingdom**: Plantae
- **Order**: Myrtales
- **Family**: Combretaceae
- **Genus**: *Terminalia*
- **Species**: *chebula*

Useful part: Fruits, Leaves and Bark

Vernacular names: Yellow Myrobalan, Haritaki, Hirada Kadukkai

Ayurvedic Properties

- **Rasa**: All but salty, mainly astringent, bitter, hot, sweet.
- **Guna**: Light, dry
- **Veerya**: Usna
- **Vipaka**: Sweet
- **Dosha**: Pacifies tridosha (Vata, Pitta, Kapha)

Chemical composition (Yadav *et al*., 2011, Gupta *et al*., 2012)

Researchers have isolated a number of glycosides from Haritaki, including the triterpenes arjunglucoside I, arjungenin, and the chebulosides I and II. Other constituents include a coumarin conjugated with gallic acids called chebulin, as well as other phenolic compounds including ellagic acid, 2,4-β-D-glucopyranose, chebulinic acid, gallic acid, ethyl gallate, punicalagin, terflavin A, terchebin, luteolin, and tannic acid. Chebulic acid is a phenolic acid compound isolated from the ripe fruits. Luteic
acid can be isolated from the bark. *T. chebula* also contains terflavin B, a type of tannin while chebulinic acid is found in the fruits.

**Pharmacological Value** *(Yadav *et al.*, 2011, Gupta *et al.*, 2012)*

Haritaki is a rejuvenative, laxative (unripe), astringent (ripe), anthelmintic, nerveine, expectorant, tonic, carminative, and appetite stimulant. It is used in people who have leprosy (including skin disorders), anaemia, narcosis, piles, chronic, intermittent fever, heart disease, diarrhoea, anorexia, cough and excessive secretion of mucus, and a range of other complaints and symptoms. Haritaki is used to mitigate Vata and eliminate ama (toxins), indicated by constipation, a thick greyish tongue coating, abdominal pain and distension, foul faeces and breath, flatulence, weakness, and a slow pulse. The fresh fruit is dried and the powdered, made into a paste and taken with jaggery is mala shodhana, removing impurities and wastes from the body. Haritaki is an effective mild purgative when taken as a powder, but when the whole dried fruit is boiled the resulting decoction is grahi, useful in the treatment of diarrhea and dysentery. The fresh or reconstituted fruit taken before meals stimulates digestion, whereas if taken with meals it increases intelligence, nourishes the senses and purifies the digestive and genitourinary tract. Taken after meals Haritaki treats diseases caused by the aggravation of Vayu, Pitta and Kapha as a result of unwholesome food and drinks. Haritaki is a rasayana to Vata, increasing awareness, and has a nourishing, restorative effect on the central nervous system. Haritaki improves digestion, promotes absorption of nutrients, and regulates colon function. Some pharmacological activities are antioxidant and free radical scavenging activity, anti-mutagenic, radioprotective activity. Also shows hepatoprotective potential, cardioprotective cytoprotective, anti-bacterial, anti-diabetic, anti-protozoal
activity effect also shown by haritaki. Haritaki has a demonstrated strong anti-inflammatory and anti-arthritic activity.


Harikati is used in many Ayurvedic formulations such as Dashmula Haritaki, Gomutra Haritaki, Triphala churna, Triphala guggule. Also Haritaki used in different Asava-Arishta and Ghrut.

**Dosage**

For Adults 1 g to 1.25 g once daily or divided equally into 2 doses after food even recommended in pregnancy.

**3.2.5. Haridra (Turmeric)** (The Ayurvedic Pharmacopoeia of India, 2002)

Turmeric is a very popular herb in Ayurveda and in Indian cooking due to its multitude of health benefits.
Botanical name: *Curcuma longa* (L.)

Classification:

- **Kingdom**: Plantae
- **Order**: Zingiberales
- **Family**: Zingiberaceae
- **Genus**: Curcuma
- **Species**: longa

Useful part: Rhizomes (tuber)

Vernacular names: Haridra, Haldi.

Ayurvedic Properties

- **Rasa**: Katu and Tikta
- **Guna**: Laghu and Ruksha
- **Veerya**: Usna
- **Vipaka**: Katu
- **Dosha**: Tridoshic at normal dosages.

Chemical composition (Aggarwal *et al.*, 2007)

Turmeric has hundreds of molecular constituents, each with a variety of biological activities. For instance, there are at least 20 molecules that are anti-biotic, 14 that are known cancer preventatives, 12 are anti-tumor, 12 are anti-inflammatory and there are at least 10 different potent anti-oxidants. The list goes on and on with addition of more research and evidences. Turmeric is a veritable pharmacy in its own right, with literally hundreds of molecules and activities on its shelves. This is also the testimony to the use of whole herbs and not just isolated molecules. And speaking of molecules, by far the
most of research in Turmeric spins around three gold-coloured alkaloids Curcuminoids: Curcumin, Demethoxy-curcumin, and Bisdemethoxy-curcumin. Most of the research done is with a 95% Curcuminoid extract of Turmeric, though in its raw state Turmeric contain only 3-5% Curcuminoids. The rhizome is 70% carbohydrates, 7% protein, 4% minerals, and at least 4% essential oils. It also has vitamins, other alkaloids, and is about 1% resin.

**Pharmacological value** (Gargevi *et al.*, 2011)

Indigestion, flatulence, poor circulation, cough, amenorrhea, pharyngitis toothache, chest pain, blood urine, hemorrhage, skin disorders, diabetes, arthritis, anemia, wounds, bruises, to strengthen the stomach and promote its action, and also as a tonic and blood purifier. Turmeric Poultices is often applied locally to relieve inflammation and pain. Turmeric acts on the imbalanced processes of digestion, metabolism and nutrition and restores the normal function of the gastro-intestinal system. It is also considered to have excellent natural antibiotic and antibacterial actions. It not only purifies the blood (Rakta dhatu shuddhi), but also warms it and stimulates formation of new blood cells (Rakta dhatu vardhaka).

**Important Ayurvedic formulations of turmeric**

Turmeric is widely used in different Ayurvedic or herbal preparations such as Asava-Arishta, Eladya Modaka, Ghrita (Tiktaka Ghrit).

**Dose** (The Ayurvedic Pharmacopoeia of India, 2000)

1-3 g of drug in powder form
3.2.6. *Woodfordia fruticosa*

Detailed review of *W. fruticosa* and its Pharmacognostic and Pharmaceutical information is given in Chapter 2.1.

3.2.7. **Jaggery** (Jagannadha Rao *et al.*, 2007)

(www.trekearth.com and www.organicfacts.net)

The scientific or technical definition of jaggery is an amorphous form of unrefined and non-distilled sugar prepared from the sap or the juice of plants that contains a considerable amount of sucrose or sugar. This includes things like sugar cane and certain palms like date palm and Palmyra. Sometimes, it is also called country sugar, since it is prepared in rural households of certain countries. It is also called molasses due to its semi-solid state. Jaggery is extensively used in different Indian cuisine and in various Ayurvedic medicines. It is a good substitute for sugar. Not only that, it comes with a bunch of its own unique health benefits. Jaggery forms the backbone of Asavas and Arishtas, fermented liquid Ayurvedic medicines.
Ayurveda explains two types of jaggery i.e.

1. Dhauta-washed/semi-refined
2. Adhauta-unrefined/unwashed

**Common names of jaggery**

Guda, Gud, Gula and gulam in Ayurveda.

**Important Ayurvedic formulation of jaggery.**


**Chemical composition of jaggery** (Harish Nayaka *et al.*, 2009)

The jaggery contains approximately 60-85% sucrose, 5-15% glucose and fructose. Along with 0.4% of protein, 0.1 g of fat and 0.6 to 1.0 g of minerals (8 mg of calcium, 4 mg of phosphorus, and 11.4 mg of iron). It is also found to contain traces of vitamins and amino acids. 100 g of jaggery gives 383 kcal of energy. In Ayurveda, jaggery is considered as the best base material for the preparation of medicines. In contrast, the white crystal sugar contains only sucrose to the tune of 99.5% without any minerals and regarded as white or sweet poison.

**Benefits of Jaggery**

- The conventional process of making jaggery does not involve any chemical agents will have all natural mineral and salts retained in it.
• Cleansing Agent: Jaggery is one of the best natural cleansing agents for our body, which effectively cleans the respiratory tracts and digestive system.

• Better Digestion: Jaggery improves digestion. Jaggery activates various digestive enzymes and itself gets converted to acetic acid, thus speeding up digestion.

• Jaggery is complex than sugar as it is made up of longer chains of sucrose.

• It is digested slower than sugar releasing slow energy.

• Unlike empty calories in sugar, jaggery is source of Fe, Ca, K and Phosphorus.

• Mineral Source: It is considered as a nutrients storehouse. It has minerals like iron, magnesium, phosphorus, potassium, etc. Jaggery is a rich source of many vital minerals that are required by the body for normal growth and functioning.

• Jaggery is used since many decades as medicine to treat dry cough, cold and asthma.

• Jaggery helps in treating conditions like indigestion and constipation.

• Jaggery helps in promoting relaxation of muscles, nerves and blood vessels, thus enhancing their functions.

• Jaggery is also a good source of magnesium, which is beneficial for the muscles, nerves and blood vessels and also helps relieve fatigue.

• Jaggery helps to relieve the most uncomfortable and painful headaches, Migranes.

• Jaggery is a rich source of iron and is therefore, very good for anaemic patients, as it increases the haemoglobin level in the blood. It helps regulate menstruation.

• Jaggery has strong antioxidant properties and protects our body cells from the damage caused by free radicals due to oxidative stress.

• Jaggery has the ability to purify blood and helps in regulating liver function.
• A mixture of jaggery and dry ginger powder (Suthi) taken with warm water can stop hiccups.

• Jaggery helps in maintaining blood pressure and also helps the body to flush all the toxins.

• Jaggery is loaded with many essential nutrients such as vitamins and minerals that play a key role in many metabolic processes.

• Jaggery has potassium that helps in reducing bloating and water retention.

3.3. Literature Review

3.3.1. Pharmaceutical and Phytochemical Review of Ashvagandharishta

Ayurvedic Pharmacopoeia of India, 2008 has described the details about Ashvagandharishta. Specifics composition, method of preparation, description, physico-chemical parameters, TLC for identification, storage, therapeutic uses and dose were well mentioned.

In Ayurvedic Pharmacopoeia of India (2008) describes the methodology for the chromatographic identification of phytocompounds from Ashvagandharishta. Method describes as follow, Measure (50 ml) and lyophilises the formulation under vacuum to remove alcohol and reconstitute to 50 ml by adding distilled water. The resulting solution is partitioned successively with n-hexane (50 ml x 3) and chloroform (50 ml x 3). Filter and concentrate the chloroform extract under vacuum and weigh. Dissolve 20 mg of residue in 1 ml of chloroform and carry out the thin layer chromatography. Apply separately 10 µl of solution prepared as above and 5 µl of standard solution prepared by dissolving 1 mg of withanolide D in 1 ml of methanol, on TLC silica plate and develop the plate to a distance of 8 cm using toluene: ethyl acetate : acetic acid (5 : 4 : 1) as
mobile phase. After development, allow the plate to dry in air and spray with anisaldehyde-sulphuric acid reagent followed by heating at 1050 for about 10 minutes and examine under ultra violet light (366 nm). It shows major spots at Rf 0.27 (dark purple), 0.44 (purple) corresponding to withanolide D, 0.61 (light grey), and 0.70 (dark brown).

Kushwaha et al., (2011) has prepared Ashvagandharishta, using Musali, YashtimadhuVA Vidari, Shatavari, Brahmi, Shankhapushpi, Daruharidra, Arjuna, Sarkara, Dhataki, Sunthi, Pippali, Nagkesara and curcuma. They have used sugar (cane sugar), jaggery and honey for the preparation of formulation and also suggested that either of it can be used in pure form, jaggery should be very old. The earthen pot or jar intended for fermenting the medicine is tested for weak spot and cracks and similarly a perfect lid is chosen. However, prepared subjected to physiochemical, phytochemical and chromatographic analysis. Parameters such as pH, specific gravity, total ash value, Acid insoluble ash, water soluble ash sulphated ash and moister content were determined. Present study has standardized by HPTLC, HPLC and pharmacokinetic profiling methods by using markers. Kushwaha et al., (2011) Ashvagandharishta was extracted with methanol and subjected to TLC using Benzene: Ethyl acetate (9:1 v/v) with iodine vapours as detecting agent. Blackish-brown spot was found with Rf 0.8. Kushwaha et al., (2011) aimed to understand the benefits of Asava-Arishta and need to sterilise them. They also commented that in current scenario there is great importance of Asava-Arishta as these products can undergo transformation during maturation, which help to enhance therapeutic activity.

Tiwari et al., (2011) prepared two different Ashvagandharishta formulations to compare its diuretic activity in experimental rats. Traditional formulation was prepared using the
procedure mentioned in Ayurvedic Pharmacopoeia of India, 2008. Ashvagandharishta using modern methodology have similar method of preparation, only *W. fruticosa* flowers were replaced with Yeast for inducing fermentation, Yeast source is not mentioned in their research article.

Singh *et al.*, (2013) has estimated naturally produced water soluble vitamins in different Asava-Arishtas such as Drakshasava, Ashokarishta, Dhashmulrishta, Khadirarishta, Lauhasava, Arjunarishta and Ashvagandharishta. This study revealed that these preparations contain water soluble Vitamins B\(_1\), B\(_2\), B\(_3\) and B\(_6\) in significant concentrations. Singh *et al.*, 2013 has used water, methanol and acetic acid in the ratio of 73:26.5:0.5 with 1.92 pentane sulphonic acid and 0.9 ml triethylamine were used in the study. Ashvagandharishta contains very less amount of Vitamin B\(_1\) and highest amount of Vitamin B\(_2\). Presence of these water soluble vitamins in different concentrations justifies the claimed benefits of these Ayurvedic preparations.

Jirage *et al.*, (2010) have standardised commercial formulations and extracts, which contain Ashvagandha as a main constituent. Extract and formulations such as *Momordica charantia* Linn extract, Ashvagandha vati, Ashvagandha churna, Ashvagandha capsule, Ashvagandha tablet and Ashvagandharishta were analysed for determination of β-sitosterol-D-glucoside. The mobile phase consisted of toluene: ethyl acetate: formic acid (5:5:0.5 v/v/v) were optimised and validated for the determination of β-sitosterol-D-glucoside in above mentioned formulations. All Ashvagandha formulations contain measurable amount of β-sitosterol-D-glucoside. Jirage *et al.*, (2010) also suggested usefulness of this method to estimate β-sitosterol-D-glucoside in different Ayurvedic or Herbals formulations.
Tiwari et al., (2012) developed and validated new HPTLC method for quantification of gallic acid and ellagic acid in Ashvagandharishta. Two different Ashvagandharishta were prepared using traditional method and modern methodology and also compared with marketed Ashvagandharishta. Traditionally Ashvagandharishta was prepared using method given in Ayurvedic Pharmacopoeia of India and Ashvagandharishta with their methodology was prepared by replacing the W. fruticosa flowers by commercially available Yeast. The developed HPTLC method was validated in terms of precision, accuracy, LOD, LOQ and specificity. The amount of gallic acid in Ashwagandharishta-T, M and its marketed formulation was found to be 0.0281, 0.0279 and 0.0280 % w/w, respectively, while ellagic acid was found to be 0.0191, 0.0189 and 0.0188 % w/w, respectively. This is the first report for quantification of gallic acid and ellagic acid from Ashvagandharishta.

Tiwari et al., (2012) developed a simple, precise and accurate HPTLC method for the determination of quercetin and rutin in Ashwagandharishta-T and Ashwagandharishta-M prepared by traditional and modern methods, respectively and also in its marketed formulations. The amount of quercetin in Ashwagandharishta-T, M and its marketed formulation was found to be 0.0021, 0.00192 and 0.00197 % w/w, respectively, while rutin was found to be 0.00469, 0.00441 and 0.00464 % w/w, respectively. The developed HPTLC method was validated in terms of precision, accuracy, LOD, LOQ and specificity. This was the first HPTLC report, which claimed, quantification of quercetin and rutin in Ashvagandharishta.

Manwar et al., (2012) developed HPLC method using DAD and ELSD detector, which was developed for simultaneous determination of Withaferine A and Withanolide A from
the roots and its formulations such as Ashvagandharishta. Ashvagandharishta formulation was simultaneously extracted with chloroform and ethyl acetate after drying these extracts were combined and again reconstituted with methanol. Retention time for Withaferin A (3.74, 3.84 min) and withanolide A (4.51, 4.60 min) with DAD and ELSD detector, respectively. Finally Manwar et al., (2012) concluded that their HPLC method is suitable for the determination of withaferin A and withanolide A from roots and herbal formulations of Ashvagandha (Withania somnifera).

Ashvagandha (W. somnifera) is one of the most important herbs that have long been considered as an excellent rejuvenator and immunomodulatory agent (Sharma et al., 1999, Chatterjee et al., 1995, Bone et al., 1996). Several formulations are prepared using Ashvagandha. It is also scientifically proven that Ashvagandha extract has strong antioxidant and anti-inflammatory effect attributed mainly to the biologically active compounds such as alkaloids (ashwagandhin, cuscohygrine anahygrine and tropine) and steroids (β-sitosterol-D-glucoside, withaferin A, withasomniferin-A, withasomidienone, withasomniferols A-C and withanone) (Rastogi et al., 1998, Abraham et al., 1975, Ali et al., 1997). In the present work, simple, precise and rapid HPTLC method for simultaneous estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A from Ashvagandharishta has been described. Previously, different methods have been reported for HPTLC analysis for individual estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A. To the best of our knowledge this is the first report on a method for concurrent analysis and estimation of these marker compounds.
3.4. Phytocompound Profile

3.4.1. β-Sitosterol-D-Glucoside

Chemical name 5, 20 alpha-Dihydroxy-6 alpha, 7 alpha-epox-1-oxowitha-2, 24

CAS Number 32911-62-9

Molecular formula C_{28}H_{38}O_{6}

Molecular Weight 470.602

Appearance White to Off-white solid

Solubility Soluble in methanol and ethanol

Melting point 305-308°C

Category Anti-convulsive, Anti-inflammatory, Antioxidant and Anti-tumor

Storage Sample should be kept at -20°C
3.4.2. Gallic acid

Chemical name: 3,4,5 Trihydroxybenzoic acid

CAS Number: 149-91-7

Molecular formula: C_7H_6O_5

Molecular Weight: 170.12

Appearance: White powder

Solubility: Soluble in methanol and ethanol

Melting point: 251°C

Category: Antioxidant, Anti-inflammatory, Antifungal

Storage: Sample should be kept at room temperature
3.4.4. Withanolide-A

Chemical name: 5, 20 alpha-Dihydroxy-6 alpha, 7 alpha-epox-1-oxowitha-2, 24 dienolide

CAS Number: 32911-62-9

Molecular formula: C_{28}H_{38}O_{6}

Molecular Weight: 470.602

Appearance: White to Off-white solid

Solubility: Soluble in methanol and ethanol

Melting point: 305-308°C

Category: Anti-convulsive, Anti-inflammatory, Antioxidant and Anti-tumor

Storage: Sample should be kept at -20°C
3.4.4. Withaferin-A

Chemical name 4b, 27-dihydroxy1-oxo-5b, 6b-epoxywith-2,24-dienolide

CAS Number 5119-48-2

Molecular formula C$_{28}$H$_{38}$O$_{6}$$

Molecular Weight 470.60

Appearance White prisms

Solubility Soluble in methanol, ethanol and dimethylsulfoxide

Melting point 252-253°C

Category Anti-convulsive, Anti-inflammatory, Antioxidant and Anti-tumor

Storage Sample should be kept at -20°C
3.5. Reported methods for chromatographic analysis of β-sitosterol-D-glucoside, Gallic acid, Withaferin-A and Withanolide-A.

Sharma et al., (2007) has developed, A simple, rapid and sensitive HPTLC method for estimation of Withaferin-A and Withanolide-A in different part of W. somnifera plants using toluene: ethyl acetate: formic acid (5:5:0.1 v/v/v) mobile phase. Quantitative evaluation of the plate was performed in the absorption reflection mode at 530 nm. This method was validated for precision, repeatability, accuracy and with excellent reproducibility. Sharma et al., (2007) finally concluded that novel method can be useful for routine analysis as well as for quality control of raw materials and herbal formulations.

Gallic acid and ellagic acid are two widely occurring phenolic compounds of plant origin, to which many biological activities including anticancer and antiviral activity have been attributed. A simple, rapid and sensitive HPTLC method was developed by Baqul et al., (2005) for simultaneous estimation of gallic acid and ellagic acid and in different herbal raw materials using toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.5 v/v/v/v) mobile phase. Detection and quantification was performed at 280 nm.

Nayak et al., (2009) has developed a densitometric HPTLC method for analysis of withaferin-A in Withania somnifera. The analyte was extracted with methanol. Withaferin-A standard and sample was spotted by use of a sample applicator. The plates were developed with toluene: ethyl acetate: formic acid 5:5:1 as mobile phase. Quantitative evaluation was performed by measuring the absorbance of the analyte zones at 200 nm in reflectance mode.
Jirage et al., (2011) developed a new simple, precise, rapid and selective high-performance thin-layer chromatographic (HPTLC) method for the simultaneous determination of Withaferin-A and β-sitosterol-D-glucoside in Ayurvedic formulations containing Ashwagandha. The mobile phase consisted of Chloroform: Methanol (8:2 v/v) and densitometric scanning was performed with Camag TLC scanner III in the reflectance absorption mode at 207 nm and operated by WINCAT software (1.3.0 Camag). The retention factors of withaferin A and beta-sitosterol-D-glucoside were 0.59 and 0.21, respectively. Linearity was obtained in the range of 5-50 µg/ml-1 for beta sitosterol-D-glucoside and 0.5-5 µg ml-1 for withaferin-A. The developed and validated method was employed for standardization of four Ayurvedic formulations for their content of the two markers. This developed and validated method can be used for the determination of batch to batch variation and routine analysis of different herbal formulations containing Ashvagandha.

Dighe et al., (2011) developed simple, sensitive HPTLC method for quantitation of β-sitosterol from herbal mixture using toluene: ethyl acetate: glacial acetic acid (6:1.5:0.1 v/v/v/v) as the mobile phase. Plates were scanned densitometrically in the reflectance-absorbance mode at 580 nm. The method was also validated for linearity, precision, limit of detection (LOD), limit of quantitation (LOQ), and accuracy. This method was found to be accurate to evaluate percent recovery of β-sitosterol at three different levels and it was found to be 98.52. This method can also be useful for the different plants and herbal formulations.

Shinde et al., (2011) developed and validated a simple, rapid, precise and accurate HPTLC method for simultaneous method for determination of withanolide A and
bacoside A in combined dosage form. The mobile phase used was mixture of ethyl acetate: methanol: toluene: water (4:1:1:0.5 v/v/v/v). The detection of spots was carried out at 320 nm using absorbance reflectance mode. The method was validated in terms of linearity, accuracy, precision and specificity. The calibration curve was found to be linear between 200 to 800 ng/spot for withanolide A. As per Shinde et al., (2011) purpose of this method was achieved and it can be useful to determine withanolide A in marketed herbal formulations.

Mohan Kumar et al., (2013) have estimated and validated gallic acid content in polyherbal formulation by HPTLC analysis, which is claimed to be cost efficient. The developed mobile phase was Toluene: Methanol: Ethyl acetate: Formic acid 30:5:55:10 (v/v/v/v). The plates were scanned at 280 nm using CAMAG densitometer with WINCAT software. Mohan Kumar et al., (2013) also concluded that their method for estimation of gallic acid was accurate, linear, rugged, simple and rapid than the reported methods and, therefore can be used for routine quality-control analysis and quantitative determination of gallic acid in formulations.

Patil et al., (2012) has developed, A novel, simple and rapid HPTLC method and developed method was developed for gallic acid estimation from Triphala churna extract. The mobile phase was Ethyl acetate: Methanol: Formic acid 8:2:1 (v/v/v). The plates were scanned at 280 nm using CAMAG densitometer with WINCAT software. This method can be useful in the estimation of gallic acid in different herbal extract, herbal formulations and in Ayurvedic formulations.
Standardization of Ayurvedic formulations: Need of the hour

Ayurveda is best known for the poly-herbal formulations. Poly-herbalism in Ayurveda is of a peculiar view, although it is challenging to explain it in terms of modern parameters (Yadav et al., 2008, Klein et al., 2013). Ayurvedic literature vouches for the phenomenon of synergism behind polyherbal formulations and admired for its clinical prophesies (Wagner et al., 2009).

However, the concerns linked with quality of the formulations have become an important issue, considering the increasing demand and large scale production of formulations (Mosihuzzaman et al., 2008). It is also a pressing need to abide by modern standards of evaluation using rapid, economic and reliable methods of evaluation (Patwardhan et al., 2003, Fabricant et al., 2001). It is therefore important to quantify number of markers in such herbal formulations through which the quality of the formulation may be assessed. The key challenge in integrating Ayurvedic medicines with the current clinical practice is lack of validated scientific data and better understanding of efficacy and safety of the herbal formulations. The need of the hour is to evolve a systematic approach and to develop well-designed methodologies for standardization of raw material as well as the final products (Vaidya et al., 2003, Marcus et al., 2002). As reviewed earlier, several workers have developed methods to assess specific markers form formulations containing Ashvagandha. Many have even attempted to achieve multiplexing by developing single method for assessing multiple compounds. However, are there is no method developed to simultaneously estimate β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A. It is therefore thought useful to develop our own method useful for standardization of Ashwagandharishta formulation.
3.6. Material and Methods

3.6.1. Materials

The plant material used for preparation of Ashwagandharishta was as per the Ayurvedic Pharmacopoeia of India. The materials were procured from Green pharmacy, Pune, India. All the herbal material was authenticated in the Department of Dravya Guna Vigyan, College of Ayurved, Bharati Vidyapeeth Deemed University, Pune, India. Withaferin A, Withanolide A and β-sitosterol D-glucoside was purchased from Natural Remedies Ltd. Bangalore, India. Gallic acid was purchased from Siga Chemicals Co. Mumbai.

3.6.2. Preparation of yeast inoculum.

Jaggery solution was prepared as a media for inoculation, adding 12 g of jaggery in 100 ml of distilled water this gives 12° Brix of jaggery solution. Prepare 12° Brix solution of jaggery then sterilized by autoclaving at 121°C for 20 min. After cooling aseptically inoculate 100 µl each of all isolated yeasts i.e. (WF1, WF2, WF3, WF4, WF5 and WF6). The flask was incubated at 30° ± 0.5°C with 110 rpm for 48 hrs. After 48 hr, Arishta formulations were inoculated with 5% v/v of yeasts consortium to initiate fermentation process in Arishta formulations.

3.6.3. Preparation of Ashvagandharishta using (W. fruticosa and consortim of isolated yeasts)

All the raw material required for Ashvagandhasrisha for 10 L as given in Table 3.2, were properly weighed, cleaned and grouped according to Kvatha dravya and Prakshepa dravya. All the grouped material was powdered as mentioned in Ayurvedic Pharmacopoeia of India. Distilled water 160 L was added to Kvatha dravya to soak
overnight and heated to boil on gas burner until the final volume was reduced to one eighth of its original volume. Allowed to cool, and was filtered through muslin cloth. The Kvath thus prepared was then transferred to clean and dry container, 23.5 Kg of jaggery was added and stirred till all jaggery was dissolved, before transferring to fermentation vessel, fine powder of Prakshepa dravya was added. The Kvath thus prepared was divided in two equal volumes (10 L each) and transferred to plastic container of 15 L capacity. One container received W. fruticosa flowers and another container was inoculated with 5% yeast consortium. The containers were shifted to dark room for maintaining its temperature conditions. The container was loosely capped for breathing. Fermentation process was frequently monitored for the signs of completion of fermentation process. Formulation prepared using W. fruticosa flowers is referred to as “Ashvagandharishta-A” and 5% yeast consortium based Ashvagandharishta formulation is referred as “Ashvagandharishta-C”
### Table 3.2. Formulation composition of Ashvagandharisha as per Ayurvedic Pharmacopoeia of India

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Herbal ingredients</th>
<th>Parts used</th>
<th>Quantity used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ashvagandha API</td>
<td>Root</td>
<td>1.953 Kg</td>
</tr>
<tr>
<td>2.</td>
<td>Musali API</td>
<td>Root</td>
<td>781 g</td>
</tr>
<tr>
<td>3.</td>
<td>Manjishta API</td>
<td>Root</td>
<td>391 g</td>
</tr>
<tr>
<td>4.</td>
<td>Haritaki API</td>
<td>Pericarp</td>
<td>391 g</td>
</tr>
<tr>
<td>5.</td>
<td>Haridra API</td>
<td>Rhizomes</td>
<td>391 g</td>
</tr>
<tr>
<td>6.</td>
<td>Daruharidra API</td>
<td>Stem</td>
<td>391 g</td>
</tr>
<tr>
<td>7.</td>
<td>Madhuka (Yasti) API</td>
<td>Root</td>
<td>391 g</td>
</tr>
<tr>
<td>8.</td>
<td>Rasna API</td>
<td>Root</td>
<td>391 g</td>
</tr>
<tr>
<td>9.</td>
<td>Vidari API</td>
<td>Root tuber</td>
<td>391 g</td>
</tr>
<tr>
<td>10.</td>
<td>Arjuna API</td>
<td>Stem bark</td>
<td>391 g</td>
</tr>
<tr>
<td>11.</td>
<td>Mustaka API</td>
<td>Rhizomes</td>
<td>391 g</td>
</tr>
<tr>
<td>12.</td>
<td>Trivrth API</td>
<td>Root</td>
<td>391 g</td>
</tr>
<tr>
<td>13.</td>
<td>Ananta API</td>
<td>Root</td>
<td>312 g</td>
</tr>
<tr>
<td>14.</td>
<td>Syama API</td>
<td>Root</td>
<td>312 g</td>
</tr>
<tr>
<td>15.</td>
<td>Sveta chandana API</td>
<td>Heart wood</td>
<td>312 g</td>
</tr>
<tr>
<td>16.</td>
<td>Rakt chandana API</td>
<td>Heart wood</td>
<td>312 g</td>
</tr>
<tr>
<td>17.</td>
<td>Vaca</td>
<td>Rhizomes</td>
<td>312 g</td>
</tr>
<tr>
<td>18.</td>
<td>Citraka</td>
<td>Root</td>
<td>312 g</td>
</tr>
<tr>
<td>19.</td>
<td>Jala API for decoction and reduced to</td>
<td>Water</td>
<td>80.00 L</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.00 L</td>
</tr>
</tbody>
</table>

**Prakshepa dravya**

<table>
<thead>
<tr>
<th>No.</th>
<th>Ingredient</th>
<th>Quantity</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.</td>
<td>Jaggery</td>
<td>11.71 Kg</td>
<td></td>
</tr>
<tr>
<td>21.</td>
<td>W. fruticosa flowers</td>
<td>Flowers (625 g)</td>
<td>500 ml 5% yeast consortium/10 L batch</td>
</tr>
<tr>
<td>22.</td>
<td>Sunthi</td>
<td>Rhizomes</td>
<td>78.1 g</td>
</tr>
<tr>
<td>23.</td>
<td>Pippali</td>
<td>Fruit</td>
<td>78.1 g</td>
</tr>
<tr>
<td>24.</td>
<td>Tvak</td>
<td>Stem bark</td>
<td>156.2 g</td>
</tr>
<tr>
<td>26.</td>
<td>Ela</td>
<td>Seed</td>
<td>156.2 g</td>
</tr>
<tr>
<td>27.</td>
<td>Tejpatra</td>
<td>Leaf</td>
<td>156.2 g</td>
</tr>
<tr>
<td>28.</td>
<td>Priyangu</td>
<td>Flower</td>
<td>156.2 g</td>
</tr>
<tr>
<td>29.</td>
<td>Nagkeshar</td>
<td>Stamens</td>
<td>78.1 g</td>
</tr>
</tbody>
</table>
3.6.4. Determination of physicochemical parameters

3.6.4.1. Preliminary evaluation of Ashvagandharishta formulations

Determination of organoleptic characteristics viz. odour, taste, colour and clarity of prepared Ashvagandharishta was carried out.

3.6.4.2. pH

Digital pH meter was used to check the pH of formulations. Prior to use the pH meter was calibrated with standard Buffer tablets of pH 4.0 and 7.0.

3.6.4.3. Determination of Specific gravity

Fill the Pyknometer (Specific gravity bottle) with the distilled solution from Ashvagandharishta formulations.

Dip the thermometer into the distilled liquid and note the exact temperature of the liquid in the pyknometer before the performed stopper is inserted then weigh the Pyknometer or specific gravity bottle with the alcoholic liquid this fill at t°C. Calculate the net weight in grams of the alcoholic liquid at t°C the Pyknometer by subtracting the weight of the empty Pyknometer. Divide the weight so obtained by the ‘Water equipment’ that is the weight in air of same volume of water content in the Pyknometer at 15°C. This gives the specific gravity of the alcoholic liquid in air at t°C/15°C. Record temperature °C to the nearest 0.500. The Specific gravity at a particular temperature (°C) found by pyknometer of specific gravity bottle method can be converted into corresponding specific gravity at 15°C/15°C.
Calculations

Specific gravity of distillate from Ashvagandharishta

Specific gravity = $W_3 - W_1 / W_2 - W_1$

3.6.4.4. Determination of Self-generated alcohol by dichromate method (spectrophotometrically) (Crowell *et al.*, 1979)

Preparation of reagent

Potassium Dichromate Reagent preparation

Potassium dichromate (34 g) is dissolved in 500 ml of distilled water in a 1L Volumetric flask. The volumetric flask is placed in ice bath and 325 ml of conc. $H_2SO_4$ is added drop wise so as to minimize the heat generated. The solution is thoroughly mixed, cooled and the volume is made to 1 liter.

Protocol for self-generated alcohol determination

One ml of fermentable broth is added to the distillation flask. Ashvagandharishta sample was diluted with addition of 25-30 ml distilled water. 50 ml volumetric flask with 25 ml of dichromate reagent is used for the collection of about 20 ml of the distillate. The flask is incubated at 60°C for 20min in warm water bath, the mixture is cooled and the volume is made to 50 ml. the standard curve is prepared by using 2 to 12 % ethanol (v/v). The blank is prepared with distilled water the amount of ethanol in the test sample is determined from the Standard curve at 620 nm.

3.6.4.5. Test for the absence of methanol
Take 1 drop of the Ashvagandharishta in a 15 ml test tube. Add 1 drop of water with 1-drop dilute phosphoric acid (10 % w/v of water) followed by 1 drop of potassium permanganate solution (1% w/v of water). Add sodium bisulphate solution drop wise until the permanganate solution is discoloured. If brown color remains add 1 drop of dilute phosphoric acid followed by 5 ml of chromotropic acid solution (5 mg chromotropic acid Na salt in 10 ml mixture of 9 ml H₂SO₄ & 4 ml water) and heat to 60°C for 10 min. If no violet color is produced it indicates the absence of methanol.

3.6.4.6. Determination of total reducing sugars (TRS) (Lane et al., 1923)

Preparation of reagent

Fehling’s solution ‘A’

Dissolve 34.64 g of copper sulphate (CuSO₄ 5H₂O) in water and make up the volume up to 500 ml.

Fehling’s solution ‘B’

Dissolve 173 g of sodium potassium tartarate in approximately 300 ml of distilled water. Dissolve separately 50 g of sodium hydroxide in 50ml of distilled water. After cooling, add it to the solution of sodium potassium tartarate and make up the volume to 500 ml with water.

Methylene Blue Solution

Dissolve 0.2 g of pure methylene blue in water and make the volume up to 100 ml with distilled water

Methodology for TRS calculation
Pipette 5 ml of fermented broth with clean and sterilized pipette, dilute by adding 40 ml of distilled water and slowly add 3 ml of conc. HCL, shake well and heat to boil. After cooling to room temperature, neutralize with anhydrous Na carbonate. Dilute it further with distilled water up to 100 ml. Rinse and fill into a burette and titrate against 10 ml of Fehling’s solution (5 ml Fehling’s ‘A’ and 5 ml Fehling’s ‘B’). Methylene blue serve as indicator, end point is change in color from deep blue to the red of the cuprous oxide.

\[
\% \text{TRS} = \frac{5.128}{B.R \times F.F \times D.F}
\]

B.R- Burette reading; F.:- Fehling’s Factor; D.F- Dilution Factor

3.6.5. Phytochemical Screening of Ashvagandharishta formulations (Khandelwal et al., 2006)

Both Ashvagandharishta formulations were extracted using various solvents in succession for preliminary phytochemical identification and to detect presence of major classes of phytochemicals like carbohydrates, protein, steroids, glycosides, flavonoids, alkaloids, tannins and amino acids.

3.7. Estimation of \(\beta\)-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A in Ashvagandharishta using the novel High performance thin layer chromatography (HPTLC) method

A novel HPTLC method for simultaneous estimation of \(\beta\)-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A was developed with an objective to standardize Ashvagandharishta. Following is the description of this method, which is published in Journal of Applied Pharmaceutical Science (Bhondave et al., 2014).
3.7.1. Instrumentation

Test samples were spotted in the form of bands of width 6 mm with a Camag 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) on a pre-coated silica gel aluminium plate 60 F254, (20×10 cm) with 250 µm thickness, (Merck, Darmstadt, Germany). The plates were prewashed by methanol and activated at 110°C for 5 min prior to chromatography. A constant application rate of 0.1 µl/s was employed and space between two bands was 6 mm. The slit dimension was kept at 5 × 0.45 mm and 10 mm/s scanning speed were employed. Linear ascending development was carried out in 20×10 cm twin trough glass chamber (supplied by Anchrom Technologists (Mumbai, India) Camag Linomat IV, Switzerland). Top of chamber was covered tightly with the lid. The optimized chamber saturation time for mobile phase was 25 min at room temperature (25°C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 9 cm for approximately 30 min. Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode for all measurements and operated by CATS software (V 3.15, Camag). Deuterium lamp emitting a continuous UV spectrum between 190 to 400 nm and tungsten lamp between 400 to 800 nm was the source of radiation.

3.7.2. Preparation of standard solutions

Standard stock solutions of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A were prepared separately by dissolving 10 mg each in 10 mL methanol to get a stock solution of 1000 µg/ml. From the resulting solutions 1 mL solution, was further diluted with methanol to obtain a working solution of 100 µg/ml.
3.7.3. Development of the optimised mobile phase

HPTLC procedure was optimised with the aim to develop as assay method. Initially, mobile phase was selected on the basis of previous reports, which was subsequently modified for better resolution of all the markers. The mobile phase containing toluene: ethyl acetate: formic acid: methanol (6:3:0.1:0.6, v/v/v/v) gave good resolution of compounds. The identities of the bands from the Ashvagandharishta extract were confirmed by overlapping the densitograms of standard with that of samples.

3.7.4. Method Validation

The HPTLC method was validated as per International conference on Harmonization (ICH) guidelines, 2005.

3.7.4.1. Linearity and range

Linearity was evaluated by applying working solutions on HPTLC plate in the range of 50-400 ng/band for β-sitosterol-D-glucoside, 150-500 ng/band for gallic acid, 50-450 ng/band for withaferin A and 50-500 ng/band for withanolide A. Peak area versus concentration was subjected to calculate least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration were estimated. Correlation coefficient alone is not suitable to prove linearity, therefore residual analysis was also performed. Sensitivity of the method was determined by estimating the limit of detection (LOD) and limit of quantitation (LOQ). They were calculated as 3.3 σ/S and 10 σ/S, respectively, where σ is the standard deviation of the response (y-intercept) and S is the slope of the calibration plot.
3.7.4.2. Selection of analytical wavelength

After HPTLC development and derivatization, bands were scanned over the range of 400-700 nm and the spectra were overlaid. All marker compounds showed considerable absorbance at 474 nm and hence was selected for further densitometric analysis (Figure 3.1).

3.7.4.3. Precision studies

Precision of the method was verified by intra and intermediate precision studies. Repeatability studies were performed by analysing three different concentrations 100, 200, 300 ng/band, of the compounds, six times on the same day. Intermediate precision of the method was checked by repeating the study with above mention concentrations on three successive days.

3.7.4.4. Accuracy studies

Both Ashvagandharishta-A and Ashvagandharishta-C were spiked with a known amount of standard markers and the percent ratios between the recovered and expected concentrations were determined. The analyzed samples were spiked with 80, 100 and 120% of 100 ng/band of β-sitosterol-D-glucoside, withaferin A and withanolide A and 200 ng/band of gallic acid (standard addition method). Accuracy was calculated by the following equation:

\[
\frac{(\text{spiked concentration} - \text{mean concentration})}{\text{spiked concentration}} \times 100.
\]
Figure 3.1. Overlain visible spectra of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A.

3.7.4.5. Robustness of the Method

Robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in the experiment. Variation in retention factor and peak areas of the four compounds was examined. Factors changed were mobile phase composition (±0.1 ml), amount of mobile phase (±5%), time from banding to chromatography (+ 10 min) and time from chromatography to scanning (+ 15 min), one
factor was varied at a time, to study the effect. The robustness of the HPTLC method was studied at concentration of 200 ng/band for β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A in triplicate. The standard deviation of peak areas and % relative standard deviation (% RSD) were calculated for each variable.

3.7.4.6. Specificity

Specificity is a measure of the degree of freedom from other active ingredients, excipients, impurities and possible degradation products. In specificity studies, β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A solutions and the Ashvagandharishta sample solutions were applied on a HPTLC plate and the plate was developed, derivatized and scanned as described above. The peak purity of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A was assessed by comparing the visible spectra of markers at peak start, peak apex and peak end positions of the band i.e., r (start, middle) and r (middle, end).

3.7.4.7. Solution Stability

The stability of standard solutions was verified after 0, 6, 12, 24 and 48 h of storage. The stability of the standard solutions was determined by comparing peak area percentage and peak purity at 200 ng/band.

3.7.5. Extraction of Ashvagandharishta formulations for estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A

Both of the Ashvagandharishta formulations were analysed with an in-house developed method for simultaneous estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A. Ashvagandharishta (50 ml) was subjected to rotary evaporation to
remove the self-generated alcohol. Distilled water (50 ml) was added to re-dissolve the residue. Extract was then partitioned successively with n-hexane (3 x 50 ml) followed by chloroform (3 x 50 ml). The chloroform extracts were combined, dried over anhydrous sodium sulphate and evaporated under vacuum. The extract was weighed and dissolved in chloroform (CHCl$_3$) for further analysis.

3.8. Results

3.8.1. Fermentation time

Ashvagandharishta formulation based on traditional methodology took around 30 days to complete the fermentation, while the process of fermentation in Ashvagandharishta using consortium of yeasts was completed within 10 days of inoculation, with no further sign of fermentation. Both formulations were filtered with muslin cloth and again centrifuged, clear formulation was bottled and kept for maturation.

3.8.2. Preliminary and physicochemical evaluation of Ashvagandharishta formulations

The visual evaluation of Ashvagandharishta formulations revealed that the formulations were pleasantly aromatic, clear and dark brown in colour. There was no frothing or release of gases. Both Ashvagandharishta formulations were with characteristics bitter taste with astringent properties. No sediment was found even after ageing for 6 months or more (Table 3.3). For Ashvagandharishta-A and Ashvagandharishta-C, final pH value was found to be 3.98 and 3.77, respectively, which was within the range given in API. Gradual shift in pH form about basic to acidic value is considered to be indicative of
progression in fermentation. This feature was quite reproducible as similar pH change was observed in our earlier formulation using same yeasts.

In our earlier work, we have prepared a model formulation using turmeric using the same yeasts consortium (Bhondave et al., 2013), pH shifts was very similar with that fermentation indicating reproducibility of pattern. Coherent with the sugar tolerant test results, the yeast consortium could successfully utilize the high amount of jaggery. Finally, the total reducing sugar in Ashvagandharishta-A and Ashvagandharishta-C were found to be 23.5% and 17.5% w/v, which was well within the range given in API. The self-generated alcohol in Ashvagandharishta-A and Ashvagandharishta-C were found to be 4.25% and 7.75% v/v respectively, according to API, the alcohol content should be in the range of 5 to 10% v/v. Specific gravity of the Ashvagandharishta-A and Ashvagandharishta-C were found to be 0.9938 and 0.9991. Methanol is absent in both Ashvagandharishta formulations.
Table 3.3. Physicochemical parameters of Ashvagandharishta formulations

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Physico-chemical parameters</th>
<th>Ashvagandharishta-A</th>
<th>Ashvagandharishta-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Appearance</td>
<td>Clear, dark brown in color. Fragrant liquid with Bitter taste. No sediment found. Without frothing or release of gases</td>
<td>Clear, dark brown in color. Fragrant liquid with Bitter taste. No sediment found. Without frothing or release of gases</td>
</tr>
<tr>
<td>2.</td>
<td>pH</td>
<td>3.98</td>
<td>3.77</td>
</tr>
<tr>
<td>3.</td>
<td>Specific gravity</td>
<td>0.9938</td>
<td>0.9991</td>
</tr>
<tr>
<td>4.</td>
<td>Self-generated alcohol (% v/v)</td>
<td>4.25 % v/v</td>
<td>7.75 % v/v</td>
</tr>
<tr>
<td>5.</td>
<td>Total reducing sugars (% w/v)</td>
<td>23.5 % w/v</td>
<td>17.5 % w/v</td>
</tr>
<tr>
<td>6.</td>
<td>Presence of Methanol</td>
<td>Absent</td>
<td>Absent</td>
</tr>
</tbody>
</table>
3.8.3. Preliminary phytochemical description

Both Ashvagandharishta formulations were subjected to preliminary phytochemical screening for the presence of types of phytoconstituents. These formulations were found to contain carbohydrates, proteins, steroids, flavonoids, alkaloids and tannins. The results of the preliminary phytochemical screening were expressed in Table 3.4.

Table 3.4. Preliminary phytochemical description of Ashvagandharishta formulations.

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Phytoconstituents</th>
<th>Ashvagandharishta-A</th>
<th>Ashvagandharishta C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n-hexane</td>
<td>Chloroform</td>
</tr>
<tr>
<td>1</td>
<td>Carbohydrate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Protein</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Steroid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Glycosides</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Flavonoids</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Tannins</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Amino acid</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.8.4. HPTLC method for simultaneous estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A in Ashvagandharishta.

3.8.4.1. HPTLC method optimization

A simple HPTLC method was optimized with the view to develop an assay method for simultaneous estimation of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A. Initially, different mobile phases were tried containing various ratios of toluene, ethyl acetate and methanol. The mobile phase consisting of toluene: ethyl acetate: formic acid: methanol in the ratio of (6:3:0.1:0.6, v/v/v/v), gave good resolution and reproducible results and was selected for further study. The optimum wavelength selected for detection and quantitation was 474 nm. The retention time for β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A were found to be 0.18 ± 0.01, 0.26 ± 0.02, 0.37 ± 0.02 and 0.57 ± 0.02, respectively (Figure 3.2).
Figure 3.2. Densitogram obtained from mixed standard solution of \( \beta \)-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A.
3.8.4.2. HPTLC method validation

**Linearity, limit of detection and limit of quantitation**

The results were found to be linear in the range of 50 - 400 ng/band for β-sitosterol-D-glucoside, 150 - 500 ng/band for gallic acid, 50 - 450 ng/band for withaferin A and 50 - 500 ng/band for gallic acid. The square of correlation coefficients ($r^2$) for the plots of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A were found to be 0.9998, 0.9983, 0.9994 and 0.9996, respectively. Linearity of the method was ascertained by residual analysis (Data not shown here). Slope was significantly different from zero. The LOD and LOQ values indicate good sensitivity of the HPTLC method Table 3.5.

**Precision**

Precision of the developed method was investigated with respect to both repeatability and reproducibility. It was performed by using sample concentration of 100, 200 and 300 ng/band. The results are shown in Table 3.5. The developed method was found to be precise as the % RSD values for repeatability and intermediate precision were less than 2%, as recommended by ICH guidelines.

**Specificity**

Assessment of peak purity of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A was done by comparing the visible spectra of marker compounds at peak start, peak apex and peak end positions of the band, which were found to be. $r$ (start, middle) = 0.998, 0.998 and $r$ (middle, end) = 0.998, 0.998 respectively. Good correlation was also obtained between markers and sample spectra of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A.
Table 3.5: Summary of Validation parameters of proposed method

<table>
<thead>
<tr>
<th>Parameters</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Linearity range</strong> &lt;sup&gt;a&lt;/sup&gt; (n=6)</td>
<td>50-400</td>
<td>150-500</td>
<td>50-450</td>
<td>50-500</td>
</tr>
<tr>
<td><strong>Correlation coefficient</strong> &lt;sup&gt;r&lt;/sup&gt;</td>
<td>0.9998</td>
<td>0.9991</td>
<td>0.9996</td>
<td>0.9997</td>
</tr>
<tr>
<td><strong>LOD</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.4299</td>
<td>17.7444</td>
<td>12.0487</td>
<td>11.0456</td>
</tr>
<tr>
<td>r^2</td>
<td>0.9998</td>
<td>0.9983</td>
<td>0.9994</td>
<td>0.9996</td>
</tr>
<tr>
<td><strong>Slope</strong></td>
<td>5.657</td>
<td>6.235</td>
<td>7.251</td>
<td>6.994</td>
</tr>
<tr>
<td><strong>Intercept</strong></td>
<td>295.3</td>
<td>429.94</td>
<td>182.01</td>
<td>188.19</td>
</tr>
<tr>
<td><strong>LOQ</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.5150</td>
<td>53.7711</td>
<td>36.5114</td>
<td>33.4717</td>
</tr>
<tr>
<td><strong>Precision (%) RSD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Intra-day</strong> (n=3)</td>
<td>0.61 - 0.73</td>
<td>0.64 - 0.78</td>
<td>0.80 - 0.89</td>
<td>0.86 - 0.91</td>
</tr>
<tr>
<td><strong>Inter-day</strong> (n=3)</td>
<td>0.66 - 0.74</td>
<td>0.66 - 0.79</td>
<td>0.81 - 0.93</td>
<td>0.87 - 0.94</td>
</tr>
<tr>
<td><strong>Robustness</strong></td>
<td>Robust</td>
<td>Robust</td>
<td>Robust</td>
<td>Robust</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>Specific</td>
<td>Specific</td>
<td>Specific</td>
<td>Specific</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentration in ng/band

<sup>b</sup> A: β-sitosterol D-glucoside, B: Gallic acid, C: Withaferin A, D: Withanolide A.

**Accuracy**

Accuracy study showed recoveries of 99.04 - 102.67, 98.77 - 101.96, 99.64 - 102.67 and 100.07 - 101.96% for β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A, respectively, indicating the reliability of the proposed densitometric method Table 3.7 and Table 3.8.
Robustness

Robustness of the given method was checked after measured alterations of the analytical parameters indicated that areas of peaks of interest and retention factor remained unaffected by small changes in the operational parameters (% RSD < 2). The summary of validation parameters of proposed method are given in Table 3.5.

3.8.5. Per cent Analysis of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A Ashvagandharishta formulations

Suggested validation methodology was applied for standardization of Ashvagandharishta formulations. The shape of the peaks was not altered by other substances present in Ashvagandharishta. The percent content of β-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A in Ashvagandharishta-A and Ashvagandharishta-C were found to be 0.0102, 0.0600, 0.0151, 0.300 and 0.0122, 0.0641, 0.0163 and 0.313%, respectively (Table 3.6). The value obtained by peak analysis after HPTLC showed that, phytocompounds in Ashvagandharishta-C were comparatively higher than in Ashvagandharishta-A.

Table 3.6. (%) content of β-sitosterol-D-glucoside, gallic acid, withaferin-A and withanolide-A in formulation A and C by HPTLC.

<table>
<thead>
<tr>
<th>Ashvagandharishta formulations</th>
<th>Drug content (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-sitosterol D-Glucoside</td>
<td>Gallic acid</td>
<td>Withaferin A</td>
<td>Withanolide A</td>
</tr>
<tr>
<td>Ashvagandharishta A</td>
<td>0.010±0.175</td>
<td>0.060±0.087</td>
<td>0.015±0.094</td>
<td>0.300±0.012</td>
</tr>
<tr>
<td>Ashvagandharishta C</td>
<td>0.012±0.174</td>
<td>0.064±0.014</td>
<td>0.016±0.080</td>
<td>0.313±0.012</td>
</tr>
</tbody>
</table>
Table 3.7. Result of recovery studies for Ashvagandharishta-A (n=6)

<table>
<thead>
<tr>
<th>Amount added a</th>
<th>Amount found a / ±S.D.</th>
<th>% Recovery / ±% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>80</td>
<td>160</td>
<td>80</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>120</td>
<td>240</td>
<td>120</td>
</tr>
</tbody>
</table>

a) a Concentration in ng/band  
b) A: β-sitosterol D-glucoside, B: Gallic acid, C: Withaferin A, D: Withanolide A.
Table 3.8. Result of recovery studies Ashvagandharishta-C ($n=6$)

<table>
<thead>
<tr>
<th>Amount added $^a$</th>
<th>Amount found $^a$ / ±S.D.</th>
<th>% Recovery / ±% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>80</td>
<td>160</td>
<td>80</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>120</td>
<td>240</td>
<td>120</td>
</tr>
</tbody>
</table>

$^a$ Concentration in ng/band

$^b$ A: β-sitosterol D-glucoside, B: Gallic acid, C: Withaferin A, D: Withanolide A.
Figure 3.3. HPTLC chromatogram of Ashvagandharishta-A

Figure 3.4. HPTLC chromatogram of Ashvagandharishta-C

3.9. Discussion

Yeast cultures isolated from *W. fruticosa* showed consistent fermentation process in Ashvagandharishta with acceptable organoleptic and physiochemical properties. Since the yeasts exhibited excellent sugar tolerance and can ferment high amount of added jaggery. All the studied physicochemical parameters were within the range as given in The Ayurvedic Pharmacopoeia of India Table 3.3.

One of the important advantages of using our yeast consortium is dramatic reduction in time required for fermentation. The consortium based fermentation was completed within 10 days while the fermentation initiated using *W. fruticosa* flowers took almost 30 days. This feature may be industrially exploited where faster fermentation can translate into more productivity form the same setup available.

Another advantage of faster fermentation time is avoidance of contamination. Asava-Arishta fermentation is designed to minimize contamination by using high sugar content and employing sugar tolerant yeasts for fermentation. However, fungal contamination is of common occurrence and that can potentially spoil commercial Asava-Arishta batches. Unwanted fungal contamination shows cotton like growth that forms a crust on the surface and finally spoils the formulation with undesirable properties like acidic taste, bad colour and offensive odour. These problems could be solved by using 5% inoculum of consortium where the yeast cells outnumber the contaminating microbes and jump-start fermentation. Once alcohol production begins, contamination is potentially ruled out.

Another common problem with Asava-Arishta production is incomplete fermentation, which often leads to less percentage of alcohol. Lesser alcohol content may leads to poor
extraction dynamics of phytocompounds, besides providing a window of opportunity for contaminating microbes to grow.

Batch to batch variation is another awkward issue often faced by the manufacturers. Variations in quality of raw materials and fermenting microbes are the two principal source of this variation. Since the batch of flowers used each time is different the yeast flora associated with the flowers is also different. This gives rise to unstandardized parameters in fermentation and sets in the batch to batch variation. These problems could be circumvented with use of yeasts consortium isolated from *W. fruticosa*. Due to defined yeasts used for Asava-Arishta batch to batch variation could be minimised can be overcome with predictable fermentation time and quality.

The HPTLC densitometric method developed for the simultaneous determination of \(\beta\)-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A was validated as per ICH guidelines. The low % RSD values (below 2%) obtained in intra-day and inter-day conditions revealed that the method is precise for the determination of \(\beta\)-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A. Specificity of the method was ascertained by spectral matching of \(\beta\)-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A standards with respective peaks in Ashvagandharishta sample. The % recovery values studied at three levels (80%, 100% and 120%) speaks about accuracy of the method. The low % RSD values of peak areas obtained by slight changes in composition and volume of mobile phase showed that the method is robust. The method proved to be useful in determination of \(\beta\)-sitosterol-D-glucoside, gallic acid, withaferin A and withanolide A from Ashvagandharishta prepared by using yeast consortium, *W. fruticosa* flowers or any marketed Ashvagandharishta. The developed HPTLC method
will be useful to check the majority of important compounds in Ashvagandharishta, which has good commercial potential.

Both of the Ashvgandharishta formulations prepared by using *W. fruticosa* flowers or the consortium of isolated yeasts revealed equivalent levels of marker compounds, suggesting similarity in the process of fermentation. The most noticeable differences between Ashvagandharishta A and Ashvagandharishta C were time taken for fermentation and alcohol percentage within the fermentation time. Both of these parameters are closely linked with the activity of yeasts, which is strongly in favour of Ashvagandharishta C. This study shows clear advantages of using yeast consortium for Asava-Arishta fermentation with equivalent or perhaps better quality of final product.

In this chapter, we concluded that, utility of the yeasts isolated from *W. fruticosa* in Asava-Arishta fermentation and in producing Ashvagandharishta with excellent quality. We have developed a method for simultaneous estimation of marker compounds and employed that method to validate our formulations. The next chapter deals with pharmacological testing of Ashwagandharishta C.
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