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Research paper

Antioxidant efficacy of different formulae utilized in the yakrut roga to Non–Alcoholic Fatty Liver Disease

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ABSTRACT

Context: Liver diseases, particularly Non-Alcoholic Fatty Liver Disease (NAFLD), represent a significant worldwide public health challenge due to their prevalence and severe complications. It represents a spectrum of liver conditions ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), leading to the development of liver fibrosis (scarring) and cirrhosis (irreversible scarring) as the final stage. Oxidative stress plays a crucial role in NAFLD pathogenesis, prompting the exploration of antioxidant therapy as a potential treatment avenue. ***Objectives:*** This study explores the antioxidant potential of three Ayurvedic formulations *phalatrikadi kwatha*, *arogyavardhana vati*, and *triphala guggulu* in the context of NAFLD management. ***Methods:*** Methods involved meticulous preparation of aqueous extracts followed by in vitro assays to quantify Total Polyphenolic Content (TPC), Total Flavonoid Content (TFC), as well as antioxidant activities using 1,1-diphenyl-2-picryl hydrazyl (DPPH) and 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activities, and Oxygen Radical Absorbance Capacity (ORAC) assays. ***Results:*** Results indicate substantial antioxidant activity in all three formulations, with *Phalatrikadi kwatha* exhibiting the highest values across assays. Notably,

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these formulations contain potent bioactive compounds such as phenolics and flavonoids, known for their hepatoprotective and anti-inflammatory properties.
Conclusion: The study underscores the therapeutic potential of these Ayurvedic preparations in addressing NAFLD and other health conditions. Further research is recommended to explore additional extracts and concentrations, enhancing the understanding of their efficacy and expanding their therapeutic applications in contemporary healthcare. These findings highlight the relevance of Ayurveda medicine in modern healthcare paradigms and emphasize the importance of continued research in herbal and mineral preparations for human health and disease management.

Keywords*:* Antioxidant therapy, Ayurveda, Herbal preparations, NAFLD, Oxidative stress

INTRODUCTION

Liver diseases (LD) play a significant global health concern due to their high prevalence, severe complications, and impact on the health system. Chronic liver disease (CLD) refers to long-term damage and progressive dysfunction of the liver. It encompasses a range of conditions that can cause progressive liver injury, leading to the development of scar tissues and irreversible scarring named cirrhosis as the final stage (Sharma & Nagalli, 2023). In 2016, it ranked as the eleventh most common cause of death and the fifteenth most prevalent cause of illness worldwide, posing significant risks to health and mortality globally. In the year 2017 deaths among men and women were approximately two - third and one–third (Cheemerla & Balakrishnan, 2021). Among them, Fatty liver disease (FLD) stands out as among the liver conditions most prevalent (Moon et al., 2020) and it is also known as hepatic steatosis (HS). FLD represents a widespread and potentially severe condition marked by the buildup of excessive fat in the liver. FLD comprises two separate classifications, alcoholic fatty liver disease (AFLD) and non-alcoholic fatty liver disease (NAFLD). ALFD arises from excessive alcohol intake, whereas NAFLD develops in individuals with minimal or no alcohol consumption (Enjoji et al., 2013).

AFLD mainly depends on the volume and duration of alcohol consumption. It presents a wide array of clinical and histological manifestations, encompassing steatosis (characterized by fat accumulation in liver cells without inflammation) as well as in some instances, progressing to steatohepatitis, which entails inflammation and damage to liver cells (Méndez-Sánchez et al., 2005). Zelman and co-workers stated that obesity, overweight, and metabolic dysfunction are closely related to NAFLD (Idalsoaga et al., 2020), and in adults, it presents two distinct histological entities, non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver (NAFL). Moreover, NAFLD stands as a globally prevalent chronic liver ailment with high susceptibility (Ferramosca et al., 2017). Among them, NASH contains features of inflammatory changes and hepatocellular injury. The other one is without features of NASH, and it is also known as simple steatosis. According to the literature studies it can be identified that NASH is a progressive condition and can develop up to liver cirrhosis and hepatocellular carcinoma. Additionally, it's noteworthy that certain instances of NAFL may evolve into NASH over time (Brown & Kleiner, 2016).

Oxidative stress (OS) plays a crucial role in both the onset and progression of NAFLD and it arises from an unevenness between the generation of reactive oxygen species (ROS) and the body's capacity to counteract them with antioxidant

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defenses, and it is highly responsible for healthy cell functions (Andrea et al., 2020). Reactive oxygen species (ROS) are the key sources that induce oxidative stress within the liver during non-alcoholic fatty liver diseases and mitochondrial dysfunction, NADPH oxidase activity, elevated levels of cytochrome P450 enzymes, increased presence of fatty acids leading to lipid peroxidation, chronic inflammatory reactions, and endoplasmic reticulum stress are the contributing factors (Rolo et al., 2012). Also, it is important to identify the significance of OS in the evolution and advancement of NAFLD (Rolo et al., 2012).

Antioxidant therapy emerges as a promising treatment for NAFLD addressing OS as a key factor in its development. By utilizing antioxidants, it aims to mitigate oxidative pressure and restore equilibrium, thereby reducing its detrimental impacts on individuals. It also assumes a pivotal role in combating oxidative stress, and it is identified as a significant state linked to liver injury in fatty liver disease by neutralizing ROS and reducing oxidative stress. Also, antioxidants help alleviate cellular damage, inflammation, and fibrosis in the liver (Li et al., 2015). Most common antioxidants used in antioxidant therapy are classified under several categories including vitamins, minerals, and plant materials.

In Ayurveda herbal and mineral preparations are commonly used as treatments and under the wide range of herbal medicines, it mostly utilizes roots, leaves, barks, fruits, and flowers in various forms including powders, decoctions, pastes, pills, and oils. Also, the mineral preparations utilized in various Ayurvedic formulations, contain *bhashma*. The *bhasmas* are unique preparations prepared after the purification and processing of minerals and metals. These, Ayurveda medicinal plants and herbo-mineral preparations provide rich natural sources of antioxidants that have potent antioxidant qualities, free radical scavenging as well and anti-inflammatory which are beneficial for health including managing various conditions of the liver specially NAFLD (Li et al., 2014).

Among the herbal and mineral preparations widely used to treat NAFLD, following three preparations, *Phalatrikadi kwatha* (PK) (Table 1), *Triphala Guggulu* (TG) (Table 2), and *Arogyavardhana vati* (AV) (Table 3), specifically recommended by the practitioners and under the gastro-intestinal diseases of Ayurveda authentic texts. PK formulation has been mentioned in the context of Chakradatta (Tripathi, 2002), Sharangadhara Samhita (Srivastava, 2015) Yoga Rathnakara (Shastri, 2004), and Bhaisajya Rathnavali (Shastri, 2010). This *kwatha* contains eight ingredients that are predominantly useful in addressing Hepatocellular jaundice, Cirrhosis, and Alcohol-induced fatty liver, with similar liver-related conditions. TG formulation has been mentioned in the text of Sharanghadhara Samhita (Nagodavithana, 2001), Yogaratnakara (Shastri, 2004), and Chakradatta (Tripathi, 2002). This *guggulu* contains five ingredients that are predominantly useful in the treatment of anti-inflammatory, anti-microbial, analgesic, and hypolipidemic properties (Neelam et al., 2023). AV mentioned in the text of Rasaratnasamucchaya- *Kushta rogadhikara* (Tripathi, 2006), Bhaishajyaratnavali- *Yakrutvikara* (Kaviraj, 2012). It consisted of plant-based and mineral-based ingredients. Especially Rasaratnasamucchaya recommended AV cure all diseases (Tripathi, 2006).

MATERIALS & METHODS

Preparation of formulation: Phalatrikadi kwatha, *Triphala Guggulu*, and *Arogyavardhana vati* were meticulously crafted by the authentic texts of Ayurveda, under the vigilant oversight of the pharmacy section at the National Ayurveda Teaching Hospital in Borella and the Provincial Ayurveda Hospital in

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Table 1: Ingredients of *Phalatrikadi kwatha* (Tripathi, 2002)

Ingredients	Botanical Names	Parts Used	Mass (grams)
Haritaki	*Terminalia chebula* Retz.	Dried peri cap	6.25g
Vibhitaka	*Terminalia balarica* (Gaertn.) Roxb.	Dried peri cap	6.25g
Amalaki	*Phyllanthus emblica* L.	Dried fruit	6.25g
Amrita	*Tinospora cordifolia* (Thunb.) Miers	Stem	6.25g
Vasa	*Adhatoda vasica* Nees	Stem	6.25g
Tikta (Katuka)	*Picrorhiza kurroa* Royle ex Benth.	Rhizome	6.25g
Bhunimba	*Andrographis paniculate* (Burm.f.) Nees	Whole plant	6.25g
Nimba tvaka	*Azadirachta indica* A.Juss	Bark	6.25g

Table 2: Ingredients of *Triphala guggulu* (Nagodavithana, 2001)

Ingredients	Botanical Names	Parts Used	Mass (grams)
Haritaki	*Terminalia chebula* Retz.	Dried peri cap	5.6g
Vibhitaka	*Terminalia balarica* (Gaertn.) Roxb.	Dried peri cap	5.6g
Amalaki	*Phyllanthus emblica* L.	Fruits	5.6g
Pippali	*Piper longum* L.	Fruits	5.6g
Guggulu	*Commiphora mukul* (Stocks) Hook.	Resin	27.6g

Table3: Ingredients of *Arogyavardhana vati* (Tripathi, 2006)

Ingredients	Chemical Names/ Botanical Names	Parts Used	Mass (grams)
Shodhita Parada	Herbal Purified Mercury	Powder	1.22g
Shodhita Ghandhaka	Herbal Purified Sulphur	Powder	1.22g
Shodhita Lauha bhashma	Ash obtained from iron	Powder	1.22g
Abhra bhashma	Purified and processed mica	Powder	1.22g
Tamra bhashma	Ash obtained from copper	Powder	1.22g
Haritaki	*Terminalia chebula* Retz.	Dried peri cap	1.22g
Vibhitaka	*Terminalia balarica* (Gaertn.) Roxb.	Dried peri cap	1.22g
Amalaki	*Phyllanthus emblica* L.	Dried fruit	1.22g
Shilajatu	*Asphaltum*	Powder	3.66g
Shuddha guggul	*Commiphora mukul* (Stocks) Hook.	Resin	4.87g
Chitraka	*Plumbago indica* L.	Root	4.87g
Katukarosana	*Picrorhiza kurroa* Royle ex Benth.	Rhizome	26.84g

Meegoda. In practical application, these medicinal formulations were predominantly administered with hot water, prompting a comprehensive analysis of their aqueous extracts.

Extract preparation: To prepare the hot-water extract, a precise protocol was adhered to 50 grams of the respective preparations were meticulously placed with 150 ml of water into a round-bottom flask. This concoction was then brought to a vigorous boil over a period of 4 hours. Subsequently, the resultant extract underwent meticulous filtration utilizing Whatman filter paper with a pore size of 0.45 µm. The obtained filtrate underwent concentration through a rotary evaporator, followed by lyophilization to preserve its integrity (yielding a percentage of 12.5% w/w). The resultant extract was then diligently stored at 4°C

Antioxidant assays: Total polyphenolic content (TPC) (Singleton et el., 1999). Total flavonoid content (TFC) (Siddhuraj & Becker, 2003). 1,1-diphenyl-2-picryl hydrazyl (DPPH), (Blois, 1958). 2,2-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Pellegrini et el., 1999). Oxygen radical absorbance capacity (ORAC) (Ou et el., 2001).

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RESULTS & DISCUSSION

In recent years, due to the economic crisis, and lack of relief that can be obtained by the allopathic system of medicines for diseases like NAFLD, people turn to Ayurveda and traditional or folk medicinal treatments. Also, due to the extensive medicinal properties contained in the herbal and mineral preparations used in the Ayurveda system of medicine that has been around for thousands of years, has influenced the increase of people's inclination towards that system of medicine.

Via the Folin-Ciocalteu spectrophotometric assay, quantification of total polyphenolic content (TPC) was conducted. employing Gallic Acid equivalent (GAE) as a reference standard due to its chemical stability and accessibility. This method is based on the drop of the Folin-Ciocalteu reagent by sample polyphenols in an alkaline environment, leading to the formation of a measurable blue complex, thus enabling the estimation of polyphenolic content by comparison to the GAE. The outcome was presented as milligrams (mg) of gallic acid equivalents per gram of extract, calculated based on dry weight. The analytical findings of the phenolic content of the aqueous extract of *Phalatrikadi kwatha*, *Arogyavardhana vati*, and *Triphala guggulu* is represented in Table 4.

Table 4 Total Phenolic content of *Phalatrikadi Kwatha, Arogyavardhana vati* and *Triphala guggulu*

Sample	Total Phenolic Content (mg gallic equivalents/g of drug)
Phalatrikadi kwatha	1.31± 0.05
Arogyavardhana vati	0.25± 0.00
Triphala guggulu	1.12± 0.05

The data is presented as mean ± SEM for both TPC and TFC, with a sample size of n = 4 for each parameter.

Quantification of total polyphenolic content (TPC) was conducted via the Folin-Ciocalteu spectrophotometric assay, employing Gallic Acid equivalent (GAE) as a reference standard due to its chemical stability and accessibility. This technique is predicated on the decrease of Folin-Ciocalteu reagent by sample polyphenols in an alkaline environment, leading to the formation of a measurable blue complex, thus enabling the estimation of polyphenolic content by comparison to the GAE. The result was stated as milligrams (mg) of dry weight basis, gallic acid equivalents per gram of extract. Analytical data on the flavonoid composition of the aqueous extract from *Phalatrikadi kwatha*, *Arogyavardhana vati*, and *Triphala guggulu* are presented in Table 5.

Table 5. Total Flavonoid Content of *Phalatrikadi Kwatha, Arogyavardhana vati* and *Triphala guggulu*

Sample	Total Flavonoid Content (mg quercetin equivalents/g of drug)
Phalatrikadi kwatha	0.026±0.001
Arogyavardhana vati	ND
Triphala guggulu	0.043± 0.001

The data is presented as mean ± SEM for both TPC and TFC, with a sample size of n = 4 for each parameter.

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1,1-Diphenyl-2-picryl hydrazyl (DPPH) assay relies on the principle that antioxidants can donate electrons to reduce the stable free radical leading to a change in color from purple to yellow which is measurable spectrophotometrically. In this assay, the DPPH radical solution is mixed with the test sample, and the reduction in absorbance at 517 nm is monitored as time progresses. The amount of a color change specifies the test sample's capacity to neutralize the DPPH radical. Trolox is a vitamin E analog that is soluble in water and is frequently employed as a standard reference compound for assessing the antioxidant capacity of test samples. 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), assay is a widely utilized technique to assess the antioxidant potential of compounds. Based on the antioxidant properties, transforms the blue-green ABTS radical cation into its colorless form. The decrease in absorbance at 734 nm, triggered by this reduction, correlates directly with the antioxidant potency of the sample. The assay is performed by mixing the ABTS radical cation solution with the test sample, and the decrease in absorbance is measured spectrophotometrically. Trolox, a derivative of water-soluble vitamin E, serves frequently as a benchmark compound to gauge the antioxidant potential of test samples. Results are expressed as trolox equivalents, representing the concentration of trolox required to produce the same level of ABTS radical scavenging activity as observed with the test sample. This assay provides valuable information about the ability of compounds to counteract free radicals and provide defense against oxidative stress. The analytical data of DPPH and ABTS radical neutralizing activities for the watery extract from *Phalatrikadi kwatha*, *Arogyavardhana vati*, and *Triphala guggulu* are presented in Table 6.

Table 6. 1,1-diphenyl-2-picryl hydrazyl (DPPH) and 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activities of *Phalatrikadi Kwatha Phalatrikadi kwatha*, *Arogyavardhana vati*, and *Triphala guggulu*

Sample	DPPH IC_{50} (µg/ml)	ABTS IC_{50} (µg/ml)
Phalatrikadi kwatha	8.01± 0.33	6.24± 0.03
Arogyavardhana vati	44.89± 0.38	18.75± 0.09
Triphala guggulu	17.01± 0.59	15.32± 0.08

The data is depicted as mean ± SEM for DPPH, ABTS, and ORAC assays, with a sample size of n =3 for each. Respectively, The IC_{50} values for Trolox in DPPH and ABST assays are 11.01 ± 0.09 µg/ ml and 4.37 ± 0.04 µg/ ml.

ORAC assay used to measure antioxidant activity by neutralizing free radicals compared to Trolox equivalents. Table 7 represents the analytical data of ORAC assay of the aqueous extract of *Phalatrikadi kwatha*, *Arogyavardhana vati*, and *Triphala guggulu*

During the past few years, researchers have extensively documented the phytochemical components of numerous medicinal plants (Kokate et el., 2006) (Nadkarni, 1976). Among these constituents, alkaloids, flavonoids, and phenolic combinations are recognized as the most important bioactive compounds in medicinal plants (Sayed & Mukundan, 2005) (Devendra et al., 2012). The difference is based on various reasons and the study highlights the importance of assay methodology in determining the reported levels associated with phenolic and flavonoid compounds within herbal combinations.

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Table 7 Oxygen radical absorbance capacity assays of *Phalatrikadi Kwatha, Arogyavardhana vati*, and *Triphala Guggulu*

Sample	ORAC (mg Trolox equivalents/g of drug)
Phalatrikadi kwatha	97.19 ± 0.61
Arogyavardhana vati	61.97 ± 7.51
Triphala guggulu	85.99 ± 0.61

All the data is denoted as mean ± SEM. DPPH, ABTS along with ORAC assays; sample size n =3 each. Respectively, IC_{50} Trolox for DPPH and ABST: 11.01 ± 0.09 µg/ ml and 4.37 ± 0.04 µg/ ml.

Furthermore, the findings demonstrate the utility of multivariate statistical analysis techniques in validating and interpreting complex chemical data, providing insights into the clustering patterns of medicinal plants and the impact of extraction solvents on their chemical profiles (Larson, 1988). Phenolic compounds and flavonoids are renowned for their ability to act as antioxidants and as well as scavenge free radicals, as documented in previous studies (Larson, 1988) (Williams et al., 2004). Their mechanism of action involves mitigating the effects of free radicals using their interconnected ring structures and hydroxyl groups (Amic et al., 2003). The potent antioxidant capacity of PHAF could be linked to the considerable presence of polyphenols and flavonoid constituents within it. Polyphenols and flavonoids, two types of plant metabolites, show great promise in regulating various biological functions including anti-diabetic, antioxidant, hepatoprotective, antimicrobial, and anti-inflammatory actions, as indicated by previous studies (Abbas et al., 2017; Karak, 2019). Hence, the abundance of polyphenols and flavonoids in the aforementioned samples likely plays a pivotal role in their demonstrated hepatoprotective and anti-inflammatory activities. These bioactive properties hold particular significance in addressing fatty liver disorders, particularly '*yakrut roga*' as described in Ayurveda. The DPPH assay relies on a stable synthetic radical, maintaining its stability across various solvents, including water, methanol, or ethanol (Wani et al., 2018). Interaction with water-soluble antioxidants, acting as scavengers of proton radicals or providers of hydrogen, induces a conversion in the straw hue from the deep violet color of DPPH, detectable at 517 nm (Alam et al., 2013) (Saeed et al., 2012). Conversely, the ABTS+ assay encompasses the generation of a blue/green ABTS+ chromophore through the oxidation of ABTS with potassium persulfate. Antioxidants capable of hydrogen donation reduces the blue/green color of ABTS+, quantifiable spectrophotometrically at 745 nm (Hoque et al., 2011). Notably, while the DPPH assay predominantly targets hydrophilic antioxidants, the ABTS+ assay assesses both antioxidants soluble in water and fat (Re et al., 1999). Widely utilized for quantifying antioxidant activity by measuring the ability to scavenge free radicals, these assays offer valuable insights into the efficacy of antioxidant compounds. DPPH and ABTS+ values of the three samples, showcasing notably high antioxidant activity, as evidenced by IC50 values below 10 µg/ml. Noteworthy is the observation that IC_{50} values of ABTS+ for aqueous extracts were considerably lesser than those of DPPH values. This suggests the prominent involvement of lipophilic antioxidants alongside hydrophilic counterparts in scavenging free radicals. The ORAC assay was used to assess a substance's capacity to counter peroxyl radicals produced from the natural degradation of 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) (Alam et al., 2013) (Pisoschi & Negulescu, 2011). In this assay, the ORAC value

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serves as a direct indicator of the antioxidant power of a substance. Specifically, it quantifies the capability of the substance to prevent the corrosion of a fluorescent probe by peroxyl radicals, thereby reflecting its capacity to neutralize oxidative pressure and defend cells from harm induced by ROS. Through this method, compounds with higher ORAC values demonstrate greater antioxidant efficacy, highlighting their potential therapeutic utility in mitigating oxidative stress-related pathologies.

CONCLUSION

Based on the findings presented, it is imperative to expand the analysis to include various extracts, including methanol, and explore different concentrations. *Phalatrikadi kwatha, Arogyavardhana vati,* and *triphala Guggulu* are commonly utilized in Ayurveda, not only for liver diseases but also for other ailments. The results indicate substantial antioxidant activity in these compounds, suggesting their potential therapeutic efficacy in treating patients with *yakrut roga* (fatty liver disorder) and other health conditions. This underscores the beneficial implications of these medicines for human health. Further research investigating additional extracts and concentrations is warranted to fully elucidate their therapeutic potential. In addition to its implications for the scientific community, substantial significance for the Ayurveda community and global healthcare at large. The documented antioxidant activity of the preparations not only validates their traditional use in Ayurveda but also highlights their potential. This research reinforces the credibility of Ayurvedic medicine, providing empirical evidence to support its efficacy in addressing contemporary health challenges. Furthermore, the call for further research to explore different extracts and concentrations demonstrates a commitment to advancing Ayurvedic knowledge and enhancing the therapeutic potential of these formulations. As Ayurveda gains recognition and acceptance on the global stage, studies like these contribute to its integration into mainstream healthcare systems, offering promising solutions for improving human health and well-being worldwide. Thus, this is a valuable resource not only for the Ayurveda community but also for healthcare practitioners and policymakers seeking evidence-based approaches to holistic patient care and disease management. Given the prevalent occurrences of cancer and metabolic diseases in contemporary society, herbal preparations like those investigated in this study hold promising potential in human health. These preparations may offer beneficial implications in treating and preventing conditions such as cancer, cardiovascular disease, and other pathologies by mitigating lipid oxidation and inhibiting oxidative chain reactions. Hence, it is imperative to continue research endeavors aimed at enhancing the quality of these valuable herbal and mineral preparations. Furthermore, identifying and studying additional medicinal substances with similar properties is crucial for expanding our understanding of their therapeutic benefits and applications.

DECLARATION OF CONFLICT OF INTEREST

No conflict of interest to declare.

DECLARATION OF HONOUR

We declare on our honor that our results are not fake and made up.

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