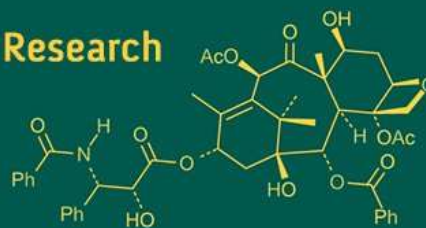
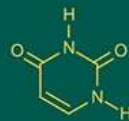
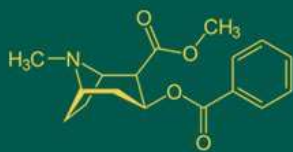


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; 10(6): 76-82
www.biochemjournal.com
Received: 01-04-2026
Accepted: 05-05-2026

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Formulation of a piperine (*Piper longum* L.) based oral herbal immune booster augmented with vitamin C and E

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i6a.8560>

Abstract

Piperine (*Piper longum* L.) increases the bioavailability of various pharmaceutical drugs as well as enhances the absorption of numerous nutrients in humans without causing any adverse effects. Additionally, piperine, known for its antioxidant properties by inhibiting free radicals and reactive oxygen species (ROS), also exhibits antifungal and antibacterial properties. The present investigation was aimed to develop an oral formulation incorporating piperine, Cherry juice for vit C, and Almond nut for vit E (alpha tocopherol), with a subsequent evaluation of its stability. The formulation had showed a density of 1.12 g/ml, a viscosity of 46.29 cp, a specific gravity of 0.927, 5.64 pH, and globular particle size of 1.137 μ m. The formulation contained 919.12 μ g/10 ml of Vitamin C and 148.73 μ g/10 ml of Vitamin E, supplemented with 4.44 g/10 ml of isolated piperine as the active ingredient.

Keywords: Piperine, vitamin, formulation, stability, viscosity, particle size

Introduction

A crucial attribute essential for successful and effective pharmaceutical formulation is the drug's adequate water solubility. The rate-limiting parameters for absorption of drugs in the gastrointestinal system include drug solubility and its dissolution rate. In the formulation of liquid dosages of drugs, a primary concern is the limited water solubility of the drugs. One of the optimal methods for achieving the efficient solubilization of pharmaceuticals is co-solvency. Co-solvents are combinations of miscible solvents that are frequently used with water to modify the solubility of drugs that are poorly soluble in aqueous phase by lowering the interfacial tension between the hydrophilic solution and non-polar solute (Yalkowsky and Roseman, 1981) [28]. Ethanol, Glycerin, Dimethylacetamide, Dimethyl sulfoxide (DMSO), Polyethylene glycol and Propylene glycol are the frequently utilized co-solvents in the development of pharmaceutical formulations (Babu *et al.*, 2008) [2].

Medicinal plants have been reported to use in the treatment of diseases of human as well as in formulation of drugs. These naturally occurring phytochemicals are termed as secondary metabolites as it is produced in the plant as a preventive and curative measure against diseases (Ezzat *et al.*, 2019) [7]. The traditional medical practitioners of India used plants with tremendous medicinal properties such as anticancer, antimicrobial, cardioprotective, antidiabetic, analgesic, antispasmodic, antiemetic and hepatoprotective for treatments of various diseases in human.

Moreover, innovative functional foods are being developed using novel bioactive ingredients with a range of immune modulatory properties. In addition to their immunomodulatory effects, these compounds also contain antioxidant, antibacterial, and antiviral properties that have an influence on immunity (Basak and Gokhale, 2022) [3]. A piperidine alkaloid piperine isolated from the dried pepper fruits (*Piper longum* L.) has various pharmacological effects and it is slightly soluble in water. The most effective method for incorporating piperine into syrup is the co-solvency technique (Suvana and Rahaman, 2020) [23]. In light of recent research findings, piperine demonstrates strong antibacterial effects against diverse pathogens and serves as a powerful antioxidant combating free radicals.

Furthermore, it has been substantiated as an effective bio-enhancer for food ingredients. The present study was undertaken to formulate a piperine based herbal oral immune booster augmented with highly effective natural antioxidants such as Vit E and Vit. C.

Materials and Methods

Piper longum L. fruits were harvested from Instructional-cum-Research (ICR) farm of Assam Agricultural University, Jorhat-13, Assam, India; and air-dried under shade. Karonda cherries (*Carissa carandas* L.) selected for their vitamin C content and almond nuts (*Prunus dulcis*) chosen for their vitamin E content were procured from the local markets of Jorhat district of Assam, India.

Isolation and Identification of Piperine

10 gm of dried ground *Piper longum* fruits of germplasm were extracted with 95% ethanol for 5 hours by using Soxhlet apparatus. After filtering, the extract was heated to 60 °C in a water bath to concentrate. 10 ml of 10% KOH solution was added to the concentrated extract and stirred continuously; insoluble residues were separated by

filtration. Water was added drop wise to the resulting filtrate. A yellow precipitation started to form. Water was then added until no more solid was forming, and the mixture was left to stand for at least one night to precipitate out the piperine crystals. The piperine crystals obtained were collected. Further re-crystallization was done by dissolving in hot acetone: hexane (3:2) followed by kept in ice bath to afford pure piperine crystals (Swapna *et al.*, 2012) [24]. The obtained isolated piperine crystals were collected, dried and yield was recorded.

Isolated piperine was identified by thin layer chromatography analysis by comparing the R_f value with standard piperine. 1mg of both isolated and standard piperine was dissolved in 1 ml of methanol separately and from this resulting solution very small amount (in μ l) was spotted on to the precoated silica gel plate. Before analysis the plate was activated by keeping it in oven at 70 °C for 1 hour. The mixture of toluene and ethyl-acetate was used as mobile phase in the ratio of 70:30 (Madhavi *et al.*, 2009) [13]. After the TLC run the spots were identified visually by keeping the plate in the iodine chamber and R_f value was determined and compared with standard.

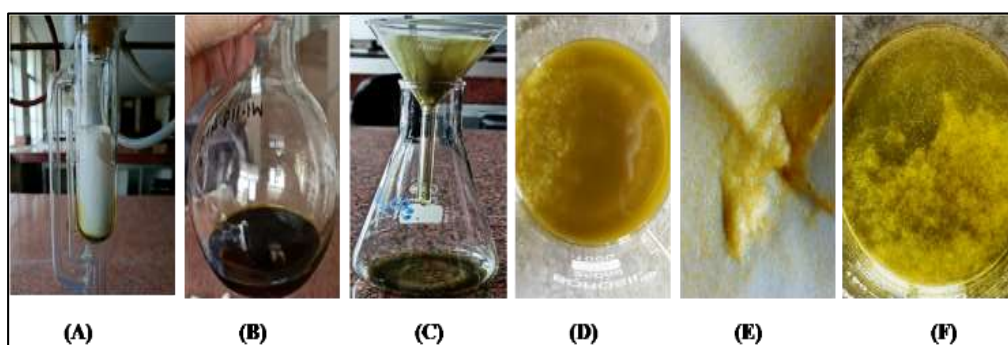


Fig 1: Isolation of piperine from *Piper longum* fruits

(A). Extraction with 95% ethanol, (B). Addition of 10% KOH, (C). Separation of insoluble residues, (D). Piperine crystal formation after adding water and leaving overnight, (E). Collection of piperine, (F). Re-crystallization of piperine.

Extraction of cherry

About 70 gm of cherry fruits were taken and cut into small pieces and 100 ml of purified water was added, homogenized and heated slowly to get the extract. The whole extract was then filtered through muslin cloths by pressing to get the juice extract. Known amount of extracted juice were used to prepare the formulation.

Estimation of vitamin C

5 g of samples were added to 50 mL of a 3% metaphosphoric acid in 8% glacial acetic acid solution and physically shaken for 15 min AOAC (1990) [1]. The same metaphosphoric acid in acetic acid solution was used to dilute the combination to 100 ml. Before being injected into the HPLC equipment, it was filtered twice: first through Whatman No. 42 paper followed by a 0.45 micron filters. It was done in triplicate for each sample. 10 μ l of the final solution was injected into HPLC chromatographic system.

HPLC operational condition

Separation of water-soluble vitamins was achieved by injecting 10 μ l extracted sample and standard solution in C18 (5 μ , 4.6 $\times$ 250 mm) HPLC column coupled to diode array detector set at 254 nm. The separation was achieved by running the HPLC instrument in isocratic mode using

Ammonium formate buffer: Acetonitrile (60:40) as a mobile phase and 10 mM Ammonium formate buffer, pH 4.0, flow rate of 0.8 ml/min for 8 minutes. Additionally, the peaks of vitamin C were determined by comparing the retention times, and their amounts were calculated by using a standard linear regression calibration equation and calculating the standard area.

Extraction of Almond oil

The almond seeds were dried in an oven and crushed into the floor with a mechanical blending mixture. Soxhlet extraction was done in triplicate for each experimental run using 15 g almond powder and refluxed with 250 mL hexane for 8 hours. After removing the hexane with a rotary vacuum evaporator, the oil was dried to constant mass in an oven at 60 °C Zhang *et al.* (2009) [29]. The percentage of oil yield was estimated using the following formula:

$$\text{Yield (\%)} = \frac{\text{Weight of oil in gram}}{\text{Weight of sample taken in gram}} \times 100$$

Estimation of vitamin E (alpha tocopherol)

In a 50 ml standard volumetric flask containing 20 ml of methanol, 500 mg of sample was placed. After making up

the volume to 50 ml with methanol, the sample was extracted by sonication for 20 minutes and centrifuged at 3700 rpm for 10 minutes. The supernatant was filtered via a 0.45 µm cellulose filter before being injected using the described HPLC technique. A total of 10 µl of the final solution was injected into HPLC chromatographic system (Nanda *et al.*, 2010 and Ubaid *et al.*, 2014) ^[16, 26].

HPLC operational condition

Separation was achieved by running the sample in Zorbax Eclipse-C18 column (5 µ, 250×4.6mm). Vitamin E (α-tocopherol) was determined by using a reserve phase high performance liquid chromatography (HPLC 1260, Infinity II) and diode array detector set at 295 nm wavelength. The mobile phase was consisting of methanol: distilled water (95:5 v/v). Flow rate was set at 1.2 ml min⁻¹ for 30 min. Peaks were calculated using retention durations in relation to recognized standards. Furthermore, the area of unknown was measured using the standard calibration curve of

multiple known standards.

Preparation of piperine based immune-booster

1. Appropriate amount of extracted almond oil and lecithin were heated in a beaker at low temperature with continuous stirring with a glass rod until a clear solution is obtained. After that glycerol was added to the mixture and stirred for 30 min with a magnetic stirrer and mixture was homogenized (Zhou *et al.*, 2010) ^[30].
2. Separately 20 mg of isolated piperine was dissolved in 2-3 g of propylene glycol by hitting and stirring and then poured in to mixture by dropwise (Suvana and Rahaman, 2020) ^[23].
3. After cooling the mixture in room temperature, the volume was made up to 50 g approximately by weight by pouring the cherry fruit extract.

By varying the different ingredients (table 1) three formulations F1, F2, and F3 were developed.

Table 1: Composition of ingredients and their quantity in different test formulation

Formulation	Lecithin (g)	Glycerol (g)	Almond oil (g)	Piperine loaded propylene glycol (g)	Sodium benzoate (g)	Cherry juice extract (g)
F1	1.5	2.5	7.6	3	0.05	35.35
F2	3	2.5	10	2	0.05	32.45
F3	3	5	5	2	0.05	34.95

Screening of different formulation

Without the use of expensive analytical tools, visual inspection is likely the easiest, least expensive, and fastest way to determine the gravitational separation of the emulsion (Guzey *et al.*, 2006; Hu *et al.*, 2017) ^[10, 11]. For screening of different freshly prepared formulation F1, F2 and F3 were stored at room temperature and visually observed for the phase separation.

Stability test of final formulation

Creaming index

The stability of the emulsion system was assessed using the creaming index (CI), which also offers details on the development of droplet aggregation McClements (2005) ^[14]. Immediately following the emulsifying procedure, the value of CI (%) for the prepared formulation was recorded in the current investigation. In order to track the stratification of the emulsions, the freshly made formulation was put into a 10 ml graduated cylinder and kept at room temperature for 3 h. Per sample, three replicates were run. CI was calculated using the formula (Xu *et al.*, 2022) ^[27].

$$\text{Creaming index (\%)} = \frac{h_1}{h_2} \times 100$$

Where,

h1 = height of the separated transparent layer

h2 = height of the total formulation taken before storage

Evaluation of final formulation

Color, odour and taste were determined by the methods described by Kumar *et al.* (2022) ^[12]. For color determination, 5 ml of the resulting syrup was poured into watch glasses, exposed to light, and the color was observed with the naked eye. For odour determination, two millilitres of finished syrup were smelled. The time gap between two smelling was fixed at 2 minutes to nullify the influence of

the previous smelling and a pinch of the final syrup was taken and tasted on tongue taste buds for the determination of taste of the final syrup.

Determination of Density

Specific gravity bottle was thoroughly cleaned with chromic acid. The bottle was rinsed at least two to three times with distilled water. The weight of empty dry bottle with capillary tube stopper was taken (W1). The bottle was filled with formulation and the stopper was placed, excess liquid from outside the bottle was wiped out using tissue paper. The specific gravity bottle with the filled formulation was weighted on analytical balance (W2). Now the weight of the test solution (formulation) was calculated in gram (W3 = W2-W1) (Patil *et al.*, 2019) ^[18]. Density of the test formulation was calculated by the formula given below and expressed in gram/ml.

$$\text{Density} = \frac{\text{Weight of liquid under cream (W3)}}{\text{Volume of liquid under cream (V)}}$$

Specific gravity

The specific gravity bottle was cleaned thoroughly with chromic acid. The bottle was rinsed at least two to three times with purified water. The bottle was rinsed with an organic solvent like acetone and dried. The weight of empty dry bottle with capillary tube stopper was taken (W1). The bottle was filled with distilled water and stopper was placed; excess liquid was wiped out from the side of the bottle using tissue paper. The Weight of the bottle with stopper and filled distilled water was recorded on analytical balance (W2). The weight of distilled water was calculated (W3 = W2-W1). The weight of the test solution (W4) was taken by repeating the method done for density determination. Specific gravity was determined by using following formula (Patil *et al.*, 2019) ^[18].

$$\text{Specific gravity} = \frac{\text{Weight of liquid under cream (W3)}}{\text{Weight of water (W4)}}$$

Determination of pH

Placed an accurately measured amount 10 ml of the final syrup in a 100 ml volumetric flask and made up the volume to 100 ml with distilled water. The solution was sonicated for about 10 minutes. pH was measured with the help of digital pH meter and pH paper (Bhagyasri *et al.*, 2018) ^[4].

Determination of globular size

Particle size was determined in the formulated fluid by using zeta sizer (Model: Nano ZS, Instruments Ltd., Malvern, UK).

Determination of viscosity and rheological behaviour

Viscosity was determined by using Anton Paar Rheometer. The gap between the two plates was adjusted to 1,000 μ m. The apparent viscosity as a function of shear rate ranging from 0.1 s⁻¹ to 100 s⁻¹ was measured.

Results and discussion

Vitamin C and Vitamin E are two dietary antioxidants that humans require. Ketoglutarate-dependent dioxygenases require vitamin C as a cofactor. Prolyl hydroxylases, for example, are involved in collagen synthesis as well as the down regulation of hypoxia-inducible factor-1, a transcription factor that regulates genes implicated in tumour formation. By scavenging reactive oxygen species

(ROS), vitamin C protects against cellular damage driven on by oxidative stress. As a powerful peroxy radical scavenger and antioxidant that breaks antioxidant chains, vitamin E suppresses the growth of free radicals in membranes and plasma lipoproteins (Traver and Stevens, 2011) ^[25]. In our current study the vitamin C content in karonda cherry was found to be 20.49 mg/100 g and the Vitamin E (α -tocopherol) content in almond nut was found to be 132.99 mg/kg of oil. Current finding was in agreement with the findings reported by Meena *et al.* (2020) ^[15], who reported that the vitamin C content in karonda cherry ranged between 10.3 to 28.0 mg/100 g. Shaheel *et al.* (2015) ^[20] also reported that vitamin C content was found to be 14.25 mg/100 g. Celik *et al.* (2019) ^[5] reported that the α -tocopherol content in different germplasm of almond oil ranged between 7.88-1085.92 mg/kg oil and 3.72-1164.36 mg/kg oil in two different provinces that support our present findings.

Screening of different test formulation (F1, F2, F3)

Three different test formulations of the piperine based immune booster were prepared with varying ingredients. F3 was the most stable of the three formulations when held at room temperature for 1 hour. The visual monitoring of phase separation revealed that there was no obvious phase separation in the F3 formulation (Fig 2), but the rest of the formulations did. The cream layer is on top, while the transparent layer on the bottom. As a result of the screening test, F3 formulation was acceptable while the others were rejected.



Fig 2: Visual monitoring of phase separation

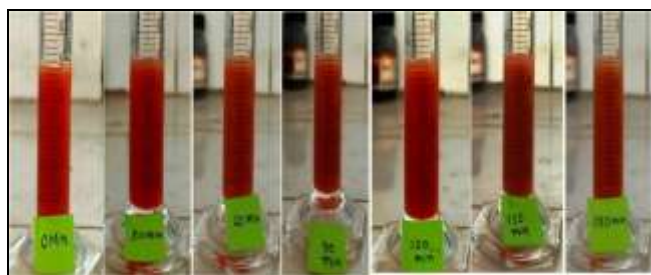


Fig 3: Assessment of creaming index

3.2 Stability of final formulation F3

When the density of the dispersed phase is lower than the density of the continuous phase, droplets move upward and separate from the continuous or aqueous phase, establishing a distinct layer on top of the continuous phase, this is referred to as creaming (Pathak, 2017) ^[17]. The emulsion loses its attracting quality, if it is creamed, and if it is not thoroughly shaken, the patient may receive an improper dosage (Rungseevijitprapa *et al.*, 2009) ^[19]. In the present

investigation the formulation showed no creaming index from 0 to 180 min of storage time (fig 3). As a result, this indicated that the generated emulsion was stable over the observed storage time length at room temperature.

Evaluation of final formulation F3

As a consequence of this research, a reddish orange-colored piperine-based with pleasant odor, sweet testing formulation with density 1.12g/ml, viscosity 46.29cp (fig 5), specific gravity 0.927, pH 5.64 and globular size of 1.137 μ m (fig 4) was developed. The formulation had a Vitamin C concentration of 919.12 μ g/10 ml and Vitamin E (α -tocopherol) of 148.73 μ g/10 ml with the inclusion of isolated piperine (4.44mg/10 ml) as an active component. Ziegenhagen *et al.* (2021) ^[31] reported that the average daily dosage of piperine in food supplements is between 5 and 30 mg; however, the marketed doses are prone to change. Ferrucci *et al.* (2010) ^[8] conducted a consumption study in India in three areas from December 2006 to July 2008 found that the average monthly for every capitaintake of black

pepper was 3.0 g to 18.5 g which was found to be 0.1g to 0.62 g per day. Piperine dosages in the range of 4-15 mg per day were commonly employed in combination studies with research lengths ranging from 4 to 17 weeks in human trial, however other studies existed in different dosages which was 60 mg per day as a dose used for 4 days trial (Delecroix *et al.*, 2017) ^[6]. Existing human studies with piperine administration, on the other hand, were primarily aimed to test the effectiveness of piperine or the effectiveness of the concomitant medications, with safety concerns being slightly addressed or reported very rarely. The majority of the trials provided no or insufficient evidence on the presence or absence of side effects, as well as no data on critical safety lab parameters and now a days piperine is frequently used and promoted in dietary supplements, among other things, as a bio-enhancer to boost the bioavailability of other substances as said by Ziegenhagen *et*

al. (2021) ^[31]. A few studies reported no negative effects when piperine (4-10 mg/day) was combined with other substances such as resveratrol, iron, curcumin etc. Therefore, in the current investigation the dose of piperine was fixed at 4.44mg per 10 ml of serving amount (10 ml) per day. Piperine increases the bioavailability of minerals nutrients including beta-carotene, coenzyme Q10, selenium, vitamin C, vitamin B6, amino acids, and curcumin, according to research by Gohil and Mehta (2009) ^[9]. In another study, Somasekhar *et al.* (2016) ^[22] measured the amount of vitamin C in rabbit blood serum using HPLC and discovered that the group of rabbits given amla plus piperine had the best bioavailability of Vitamin C in comparison to the group given amla alone. They came to the conclusion that the greater vitamin C content observed when amla and piperine were combined may have been due to piperine, which was utilized to promote bioavailability.

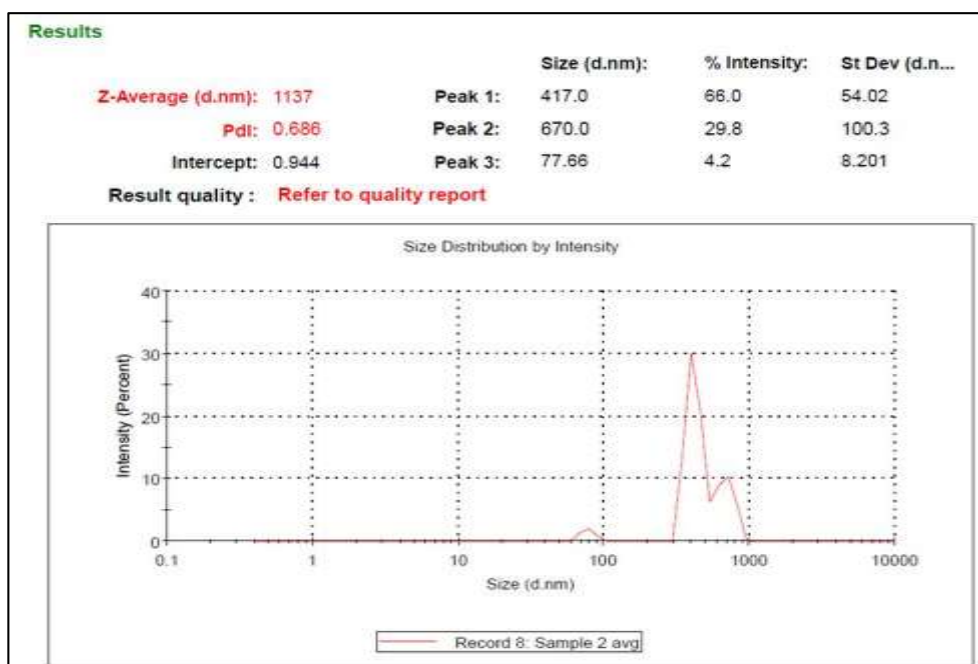


Fig 4: Determination of particle size distribution

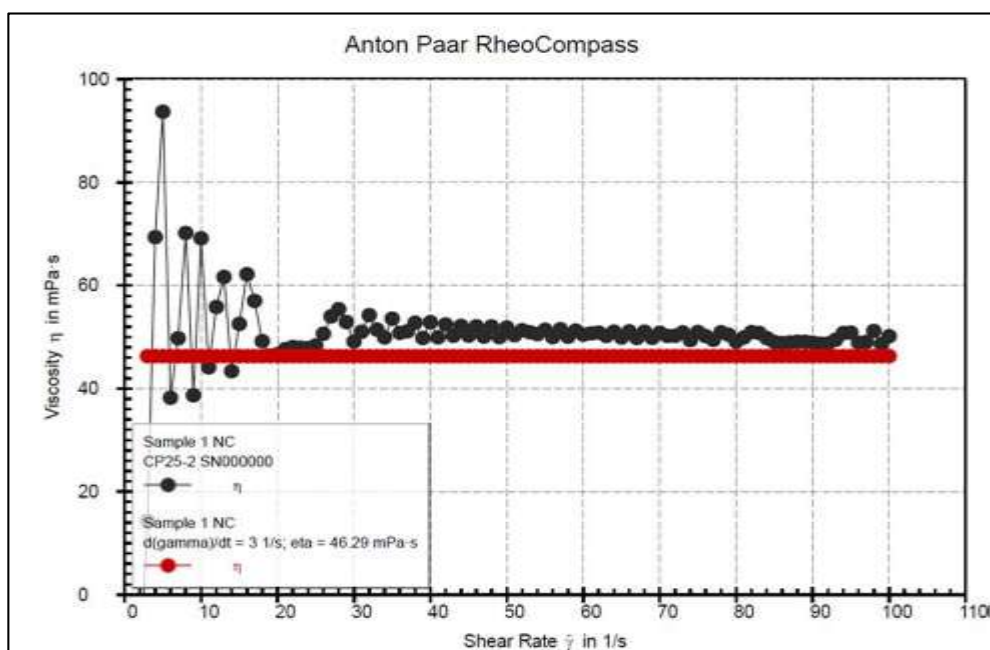


Fig 5: Determination of viscosity

Conclusion

The present study developed a stable piperine-based oral herbal immune booster enriched with natural sources of vitamin C and vitamin E from karonda cherry and almond oil. Among the tested formulations, F3 showed the best stability without phase separation and possessed acceptable physicochemical properties suitable for oral administration. The formulation contained effective levels of piperine along with antioxidant vitamins, which may synergistically enhance immune function and nutrient bioavailability. Overall, the developed formulation shows promising potential as a natural nutraceutical product; however, further *in vivo* and storage stability studies are required to confirm its efficacy and safety.

Acknowledgement

The authors are thankful to National Institute of Pharmaceutical Research and Education (NIPER) Guwahati, Assam, India for allowing to carry out Size distribution viscosity and HPLC analysis.

Conflict of Interest

The authors declare that there are no conflicts of interest relevant to this article.

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