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Enhanced Bioavailability and Efficacy of *Bacopa* Extract and Ebelin Lactone: Preparation and Biological Evaluation

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Authors' contributions

This work was carried out in collaboration among all authors. Author DRKN did the basic idea, technology, and innovation. Author SA did the extraction, formulation, phytochemical analysis. Author SP did the quantification studies. Author PS did the financial assistance. Author SU executed the experimental trials and wrote the manuscript. Author SMS performed the experimental design. Author SR did the arrangement of in vitro cell lines. All authors read and approved the final manuscript.

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ABSTRACT

Although *Bacopa monnieri* extract is well known for its cognitive and memory support, it has received minimal attention in pharmacy due to its terrible palatability and poor bioavailability. This study aimed to develop an improved, cost-effective, and eco-friendly process for the preparation of stable and highly bioavailable non-hygroscopic bacosides enriched fraction in such a manner that it contains a specific amount of Bacoside A3 and jujubogenin, and the aglycone derivative (Ebelin lactone). We prepared two compositions 1 and 2 having Bacoside A3 and jujubogenin: Ebelin

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lactone ratios 1:1 and 3:1 respectively. The efficacy of these herbal compositions was evaluated independently in comparison to the bioactivity of bacosides (20%), bacosides (50%), and Ebelin lactone. This study also aimed to find a wide range of potential activities of formulated composition, including acetylcholinesterase (AChE) inhibitor activity, anti-oxidant activity, cyclooxygenase (COX) inhibitor activity, and anti-cancer activity. We noted that both compositions worked better than parent *Bacopa* for biological activity assays but the herbal *Bacopa* Composition 1 is more potent than Composition 2. Hence, further research on Composition 1 should be explored for its potential to treat conditions such as Alzheimer's and various cancer diseases.

Keywords: Bacopa monnieri; ebelin lactone; anti-oxidant; anti-inflammatory; anti-cancer.

1. INTRODUCTION

Medicinal plants have been used historically to treat a wide range of illnesses and are an important source of pharmacologically active compounds. Traditional medicinal plants are used every day by nearly 3.3 billion people all over the world [1]. Many pharmacological components of medicinal plants are known that can be used in the creation of new drugs [2].

South and Southeast Asia are home to the *Bacopa monnieri* plant, often known as Bhrami, which is a member of the Scrophulariaceae family. It is well recognized for enhancing memory and reducing anxiety and has a long history in traditional medicine. Currently, the plant is promoted around the world as a blood sugar stabilizer and memory booster [3,4].

Recently, researchers have focused on better understanding the processes and usefulness of *B. monnieri* in treating human illnesses. *Bacopa monnieri* extracts have been investigated as a traditional medicine and used for centuries for treating a variety of illnesses, such as providing relief to patients suffering from anxiety and skin ailments particular applications include the treatment of asthma, pain, inflammation, insanity, and epilepsy [5,6]. Perhaps the most prominent medicinal claim regarding its advantages has been that it enhances memory [7]. The *Bacopa* plant, also known as a nootropic herb, aids in the healing of injured neurons, neuronal synthesis, and synaptic activity restoration, as well as improving brain function. Alkaloid brahmine, nicotinine, herpestine, Bacosides A and B, saponins A, B, and C, and triterpenoid saponins (jujubogenin and pseudojujubogenin) are major phytochemicals found in *B. monnieri* [8]. The primary bioactive components considered to be responsible for the cognitive effects of *B. monnieri* are believed to be characteristics of saponins known as "Bacosides," notably bacoside A [9,10]. Numerous *in vitro* and animal investigations have shown that the Bacosides present in *B. monnieri* strengthen the body's defenses against oxidative stress by reducing the buildup of free radicals in the brain [11]. *In vitro* testing on human lymphocytes has found that extracts from *B. monnieri* are successful in exhibiting anti-mutagenic and free radical scavenging abilities with no evidence of genotoxic activity [12]. These active constituents have been shown to have anticancer activity in addition to cytotoxic, anti-mutagenic, and anti-inflammatory properties [13]. *In vitro*, the triterpene saponins, Bacopaside I (bac I) and Bacopaside II (bac II), derived from *Bacopa* leaf extract, demonstrated a synergistic effect by inhibiting breast cancer cell migration and invasion [14,15].

B. monnieri has neuropharmacological effects and can be administered orally. The active ingredients that provide pharmacological action must be ingested and CNS active, which means that the compound must cross the blood-brain barrier and be absorbed through the gut. Poor membrane permeability *via* the blood-brain barrier and gut is most likely caused by these adverse physicochemical characteristics of the parent Bacosides. However, by satisfying four of the requirements, the aglycones (jujubogenin, pseudojujubogenin, Ebelin lactone, and bacogenin A1) demonstrated superior CNS drug-like characteristics. The blood-brain barrier allows for extensive passive lipid-mediated transit of small compounds [16]. Aglycones' tendency to permeate the brain also tends to increase as their lipophilicity rises. Orally administered substances for CNS activity is able to cross the blood-brain barrier (BBB) and be absorbed from the intestines [17,18].

This study aims to develop a formulation with maximum effectiveness of *Bacopa* extract to improve its membrane permeability and bioavailability (absorption capacity). The increased absorption is demonstrated by the herbal compositions containing Bacosides

enriched with Bacoside A3, jujubogenin, and the aglycone derivative Ebelin lactone. To ensure the drug's safety, toxicity profiling was also performed. This study also aimed to explore its effectiveness as a potent acetylcholinesterase (AChE) inhibitor, anti-oxidant, cyclooxygenase inhibitor, and anti-cancer agent.

2. MATERIALS AND METHODS

Different compositions of *Bacopa* extracts, which were a mixture of non-hygroscopic Bacosides enriched with Bacoside A3, jujubogenin, and aglycone derivative, i.e., Ebelin lactone, were assessed for their biological activities. Composition 1 was Mixture of (non hydroscopic bacosides enriched with Bacoside A3, jujubogenin) + (aglycone derivative Ebelin lactone) combined in the ratio 1:1; and Composition 2 was the mixture of (non hydroscopic bacosides enriched with Bacoside A3, jujubogenin) + (aglycone derivative Ebelin lactone) combined in the ratio 3:1 Their efficacy was evaluated independently against the bioactivity of Bacosides (20%), Bacoside (50%), and an aglycone derivative, Ebelin lactone. All the chemicals and kits used in the study were purchased from Thermofisher Scientific.

2.1 Determination of Intestinal Absorption

The Caco-2 cell line was used as an *in vitro* model for studying the intestinal permeability of the herbal composition of *Bacopa* extract enriched with Bacoside A3, jujubogenin, and aglycone derivative Ebelin lactone [19–21]. The absorption (permeability) tests were carried out using the maximum non-toxic concentration found in a preliminary set of cyto-toxicity testing. In three separate experiments, the permeability test was done at 370 °C for 2 hours in a shaking incubator with 100 RPM in the apical-basolateral (A-B) and basolateral-to-apical (B-A) directions. The samples were collected, and the amount of transported samples was determined using HPLC. To quantify the transported materials, reverse-phase chromatography using a C18 column and a UV-VIS detector was used.

Data for the permeability investigation was calculated using MS Excel. The apparent permeability coefficient (Papp) was determined using the equation:

$$(dQ / dt) / Papp (A \times C),$$

where dQ/dt (μg·s−1) is the rate of drug transport, A is the surface area of the cell monolayer (cm2), and C is the initial drug concentration on the administered side (μg·ml−1).

$$\text{Efflux ratio} = Papp (BA)/Papp (AB)$$

where Papp (BA) is the apparent permeability coefficient for the test substance transported from the basal to the apical side (secretive direction) and Papp (AB) is the apparent permeability coefficient for the test substance transported from the apical to the basal side (secretive direction) (absorptive transport) [21,22,23].

For drug absorption studies, Caco-2 cells were seeded on 35mm culture plates (80,000–100,000 cells) and allowed to adhere for 12 hours. Subsequently, the cells were treated with various herbal *Bacopa* compositions at 1 mg/ml dosage over different time intervals (0–360 min). After treatment, cell culture medium was collected, stored at -80 °C, and the cells were trypsinized, washed, and lysed in buffer. The lysed cells underwent sonication, followed by centrifugation to collect the supernatant. Protein precipitation was carried out using acetone, and the resulting mixture was centrifuged. The supernatant was then concentrated, removing acetone through vacuum concentration at 40 °C for 40 minutes. The final cell lysate was stored at -80 °C.

The chromatogram profile was obtained by HPLC analysis of the medium and cell lysate samples to determine drug absorption by the cell. The HPLC analysis was carried out with Shimadzu's Lab Solutions software and a photo diode array detector to determine the qualitative analysis of the herbal *Bacopa* compositions with the aid of an analytical standard. Bacosides and Ebelin lactone were separated using a C18-BDS column and a Restek ROC C18 column, respectively, with the appropriate solvent systems serving as the mobile phase.

2.2 Acetylcholinesterase Inhibition Assay

The amount of acetylthiocholine hydrolyzed by AChE to produce thiocholine was measured by the Acetylcholinesterase Assay Kit using DTNB. Acetylcholinesterase-generated thiocholine takes on a yellow hue when combined with 2, 5-nitrobenzoic acid. The product's color intensity at 412 nm is proportional to the amount of enzyme activity in the sample.

2.3 Determination of Radical Scavenging Activity

At various concentrations, the antioxidant activity of bacosides enriched with bacoside A3, jujubogenin, and an aglycone derivative, namely Ebelin lactone, was investigated. Ascorbic acid (1mg/100µl) was employed as a reference standard. The concentrations analyzed for *Bacopa* samples were 1 µg/µl, 2.5 µg/µl, 5 µg/µl and 10 µg/µl. After adding DPPH, the reaction mixture was stirred and incubated for 30 minutes at 25 °C. A semi-automatic analyzer was used to detect absorbance at 517 nm [24,25]. By comparing the absorbance readings of the test and control samples, the percentage inhibition was calculated.

2.4 Determination of Glutathione Reduction Assay

The herbal composition containing Bacosides enriched with Bacoside A3, jujubogenin, and the aglycone derivative Ebelin lactone, Bacosides 20%, Bacosides 50%, and Ebelin lactone was assessed for its ability to generate ROS. Ascorbic acid (1 mg/ml) was employed as a reference standard. The herbal compositions along with the Bacosides 20%, Bacosides 50% and Ebelin lactone were evaluated against 1 µg/µl concentrations of the reference standard [26]. A semi-automatic analyzer was used to measure the absorbance at 412 nm.

2.5 Determination of Anti-inflammatory Activity

A test that is quick, simple, sensitive, and dependable for screening COX-2 inhibitors is provided by the COX-2 inhibitor screening kit. This assay uses fluorometry to detect prostaglandin G2, a COX enzyme intermediate [27]. The sample was scanned for fluorescence with an excitation wavelength of 535 nm and an emission wavelength of 587 nm for 10 minutes (20 cycles) along with a time interval of 30 seconds.

2.6 *In vitro* Anti-cancer Assay

Breast cancer cells (MDA-MB-468) and colorectal cancer cells (HCT116) were analyzed for anti-cancer activity. DMSO was used as a negative control. As a positive control, doxorubicin was used. Mallick et al., (2015) method was used [28]. A Tecan Multimode reader was used to record the data and plot a non-linear regression curve against the log concentration versus absorbance

2.7 *In vitro* Cytotoxicity Assay

The Kidney Epithelial cells (Hek293T) and Skin Fibroblast cells (HFF-1) were used for cytotoxicity assay. 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml, and 1 mg/ml of each sample were tested. DMSO was used as a negative control. The standard method explained by Mallick et al., (2015) was used for the study. At 24, 48, and 72 hours, spectrophotometric readings were taken at 570 nm [28].

3. RESULTS AND DISCUSSION

3.1 Determination of Intestinal Absorption

The transport of active constituents in an apical to basolateral (A-B) direction was investigated at a concentration of 0.125 mg/ml. Composition 1 (1:1) was found to have the highest apparent permeability coefficient (Papp) in absorptive transport at 141 ± 18.7cm·s^{-1} compared to secretive direction at 77.4 ± 6.2 cm·s^{-1} and an efflux ratio of 0.54 (Fig. 1). Whereas Composition 2 (3:1) was found to have an apparent permeability coefficient (Papp) in absorptive transport (A-B) as 133 ± 26.3cm·s−1 compared to secretive direction (B-A) as 77.4 ± 10.2 cm·s−1 and with an efflux ratio of 0.57. Enriched aglycone derivative Ebelin lactone displayed an apparent permeability coefficient (Papp) in absorptive transport as 121 ± 11.3cm·s^{-1} compared to the secretive direction as 78.4 ± 8.4 cm·s^{-1} and with an efflux ratio of 0.647. Whereas Bacoside 20% and 50% showed apparent permeability coefficient (Papp) in absorptive transport as 101 ± 27.3 cm·s−1 and 113 ± 13.5 cm·s−1 respectively and secretive direction apparent permeability coefficient (Papp) as 62.4 ± 5.3 and 74.4 ± 9.6 respectively and with an efflux ratio of 0.62 and 0.66 respectively (Table 1).

The Caco-2 cells completely absorbed *Bacopa* Composition 1 (1:1) from the media 15 minutes later, as demonstrated by HPLC qualitative determination. The Caco-2 *in vitro* absorption study revealed that Compositions 1, composed of Bacosides and Ebelin lactone in a 1:1 ratio, had better membrane permeability and absorption than Bacoside 20%, Bacoside 50%, enriched aglycone derivative Ebelin lactone, and Compositions 2 (3:1), which were evaluated independently.

Table 1. Interpretation of the intestinal absorption of Bacoside 20%, Bacoside 50%, enriched aglycone derivative ebelin lactone, Composition 1 (1:1), and Composition 2 (3:1) in Caco-2 cells

	Papp X 10^{-6} (cm/s)		
Drugs	**Concentration (mg/ml)**	**Apical to Basolateral transport**	**Basolateral to Apical transport**
Bacoside 20%	0.125	101 ± 27.3	62.4 ± 5.3
Bacoside 50%	0.125	113 ± 13.5	74.4 ± 9.6
Enriched aglycone derivative Ebelin lactone	0.125	121 ± 11.3	78.4 ± 8.4
Composition 1 (1:1)	0.125	141 ± 18.7	77.4 ± 6.2
Composition 2 (3:1)	0.125	133 ± 26.3	74.4 ± 10.2

3.2 Acetylcholinesterase Inhibition Assay

An acetylcholinesterase inhibition assay showed 34% and 46% inhibition of bacoside 20% and bacoside 50% inhibited acetylcholinesterase respectively. Enriched aglycone derivative Ebelin lactone and Composition 2 were found to inhibit Acetylcholinesterase with 65% and 82% inhibition at ~ 1 mg/ml, respectively. Composition 1 (1:1), on the other hand, was found to potentially inhibit acetylcholinesterase with 85% inhibition at ~ 1 mg/ml (Fig. 2).

3.3 Free Radical Scavenging Activity of the *Bacopa* Herbal Compositions

A DPPH assay resulted as Both the *Bacopa* herbal compositions exhibited good antioxidant activity. The IC50 values of Bacoside 20% and Bacoside 50% were found to be ~ 96.12 µg/ml and ~ 84.65 µg/ml, respectively. The IC50 values of the Enriched aglycone derivative Ebelin lactone and Composition 2 were found to be ~ 45.34 µg/ml and ~ 52.12 µg/ml, respectively. Composition 1 (1:1), on the other hand, demonstrated potential anti-oxidant activity with an IC50 value of ~ 58.32 µg/ml in comparison to standard ascorbic acid with an IC50 value of ~ 7.12 µg/ml (Fig. 3).

3.4 Glutathione Reduction Assay

Bacoside 20% and bacoside 50% inhibited glutathione with 34% and 25% inhibition, respectively. The enriched aglycone derivative Ebelin lactone and composition 2 (3:1) inhibit glutathione with 13% and 27% inhibition, respectively, at ~ 1 mg/ml. Composition 1 (1:1) was found to reduce glutathione with 22% inhibition at ~ 1 mg/ml in comparison to ascorbic acid (Positive control), which exhibited only 7% glutathione reduction (Fig. 4).

3.5 Synergistic Cyclooxygenase Inhibitory Activity

Bacoside 20% and Bacoside 50% were found to inhibit COX-2 with 76% and 83% inhibition at ~ 1 mg/ml, respectively. Enriched aglycone derivative Ebelin lactone and Composition 2 (3:1) were found to inhibit COX-2 with 89.5% and 90% inhibition at ~ 1 mg/ml, respectively. Whereas, Composition 1 (1:1) was found to potentially inhibit COX-2 with 91.1% inhibition at ~ 1 mg/ml in comparison to celecoxib (Positive control), which exhibited only 69% COX-2 inhibition (Fig. 5).

3.6 *In vitro* Anti-Cancer Activity

Bacoside 20% (Figs. 6A and 7A) was found to effectively inhibit MBA-MD-468 and HCT116 cells with an IC50 value of ~ 0.2 mg/ml and ~ 0.06 mg/ml, respectively. Bacoside 50% (Figs. 6B and 7B) was found to effectively inhibit MBA-MD-468 and HCT116 cells with an IC50 value of ~ 0.03 mg/ml and ~ 0.09 mg/ml, respectively. Ebelin lactone, an enriched aglycone derivative (Figs. 8C and 9C), was found to effectively inhibit MBA-MD-468 and HCT116 cells with IC50 values of 0.210 mg/ml and 0.055 mg/ml, respectively. Composition 1 (1:1) (Figs. 6D and 7D) was found to effectively inhibit MBA-MD-468 and HCT116 cells with an IC50 value of ~ 0.11 mg/ml and ~ 0.055 mg/ml, respectively. Whereas, Composition 2 (3:1) (Figs. 6E and 7E) was found to effectively inhibit MBA-MD-468 and HCT116 cells with an IC50 value of ~ 0.11 mg/ml and ~ 0.055 mg/ml, respectively.

3.7 *In vitro* Toxicity Studies

Bacoside 20% (Figs. 8A and 9A) was found to be non-toxic against Hek293T and HFF-1 with an IC50 value of ~ 0.6 mg/ml and ~ 1.026 mg/ml, respectively. Bacoside 50% (Figs 8B and 9B) was found to be non-toxic against Hek293T and

HFF-1 with an IC50 value of ~ >10 mg/ml and ~ 2.151 mg/ml, respectively. Ebelin lactone (Figs 8C and 9C) was discovered to be non-toxic against Hek293T and HFF-1, with IC50 values of ~ 0.9 mg/ml and ~ 0.862 mg/ml, respectively. Composition 2 (3:1) (Figs 8E and 9E) was found to be safe and non-toxic against Hek293T and HFF-1 with an IC50 value of ~ 0.8 mg/ml and ~ 1.052 mg/ml, respectively. Whereas, Composition 1 (1:1) (Figs 8C and 9C) was found to be safe and non-toxic against Hek293T and HFF-1 with an IC50 value of ~ 0.9 mg/ml and ~ 1.583 mg/ml, respectively.

The major active constituents of *B. monnieri* are the steroidal saponins Bacosides A, which is a combination of Bacoside A3, *Bacopa*side II, *Bacopa*side X, and *Bacopa*saponin C. Bacoside A is thought to be a mediator of *B. monnieri*'s memory-enhancing and cognitive effects [29,30]. Nevertheless, there isn't sufficient information regarding the underlying mechanism for the bacoside components' activity. Previous studies have revealed that, the bacoside A which cater to the pharmacological action to be CNS active is improbable to be absorbed in the gut to pass through the blood-brain barrier. As a result, the Bacosides must be transformed such that the sugar units are eliminated to mediate enhanced memory and cognitive activity [31,32]. In comparison to the parent Bacosides, aglycones (jujubogenin and pseudo-jujubogenin) along with their aglycone derivatives such as Ebelin lactone have projected higher binding affinity to all CNS receptors along with improved docking to AChE in insilico models. Higher affinity to CNS receptors results in increased CNS active properties. This offers excellent oral absorption and BBB penetration of *B. monnieri.* In comparison to other ligands, Ebelin lactone also displayed high CNS receptor binding and increased BBB penetration [32]. The mixture of Bacosides with a high concentration of bacoside A3, jujubogenin, and an Ebelin lactone of an aglycone derivative has demonstrated acetylcholinesterase inhibition activity, in addition to anti-oxidant, anti-inflammatory, anti-cancer, and anti-cytotoxic characteristics.

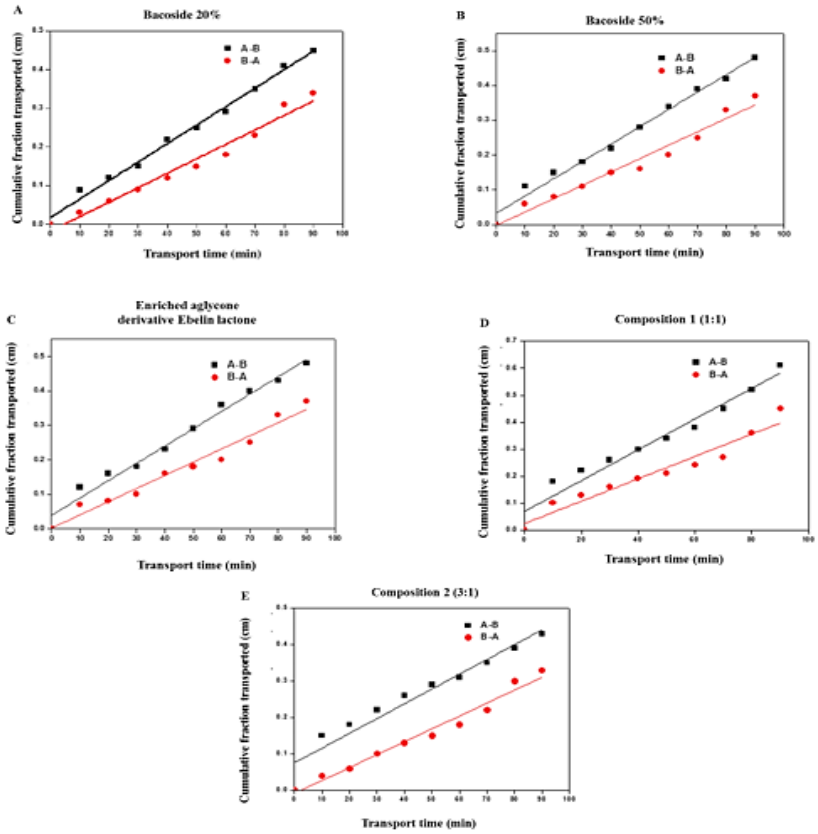

Fig. 1. Graph representing the Intestinal absorption of various *Bacopa* herbal compositions (A) Bacoside 20%, (B) Bacoside 20%, (C) Enriched aglycone derivative Ebelin lactone, (D) Composition 1 (1:1), and (E) Composition 2 (3:1) were tested for intestinal absorption

A-B- Apical to Basolateral transport, B-A- Basolateral to Apical transport

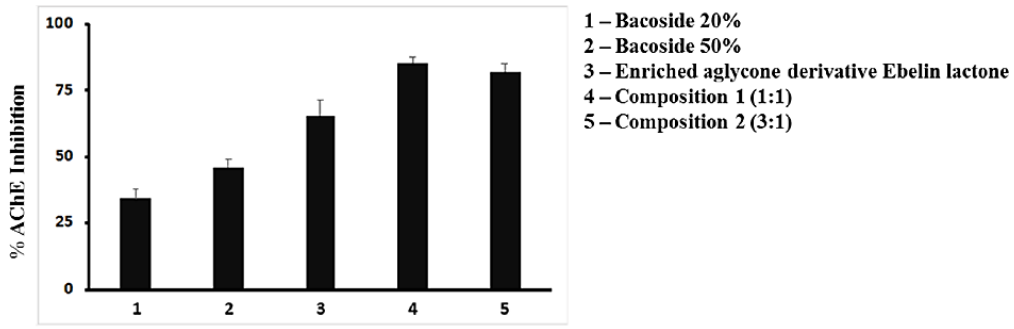

Fig. 2. Graph representing the percentage of Acetylcholinesterase inhibition for various *Bacopa* herbal compositions

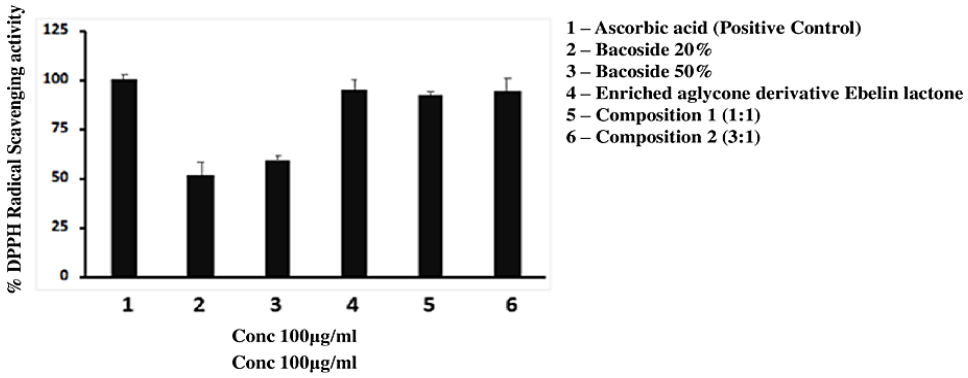

Fig. 3. Graph representing the percentage of Radical scavenging activity for various

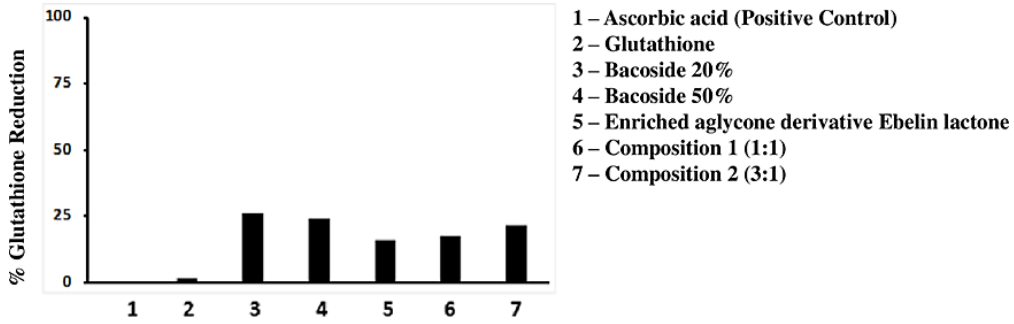

Fig. 4. Graph representing the percentage of Glutathione Reduction for various *Bacopa* herbal compositions

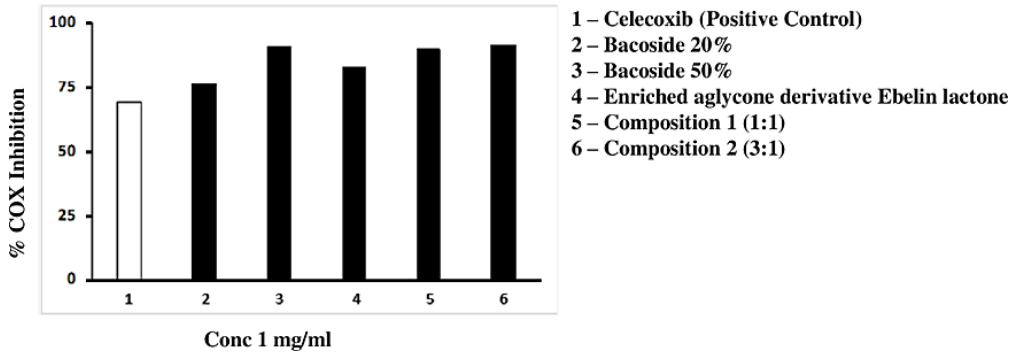

Fig. 5. Graph representing the percentage Cox-2 Inhibition for various *Bacopa* herbal compositions

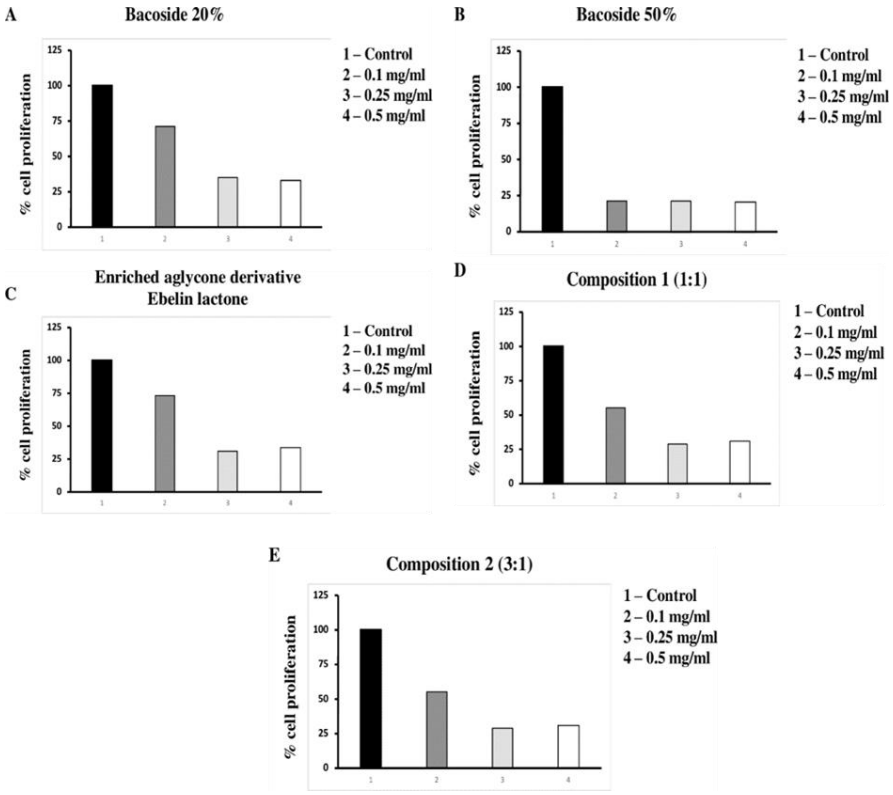

Fig. 6. Graph illustrating the percentage inhibition of proliferation of MBA-MB-468 cells

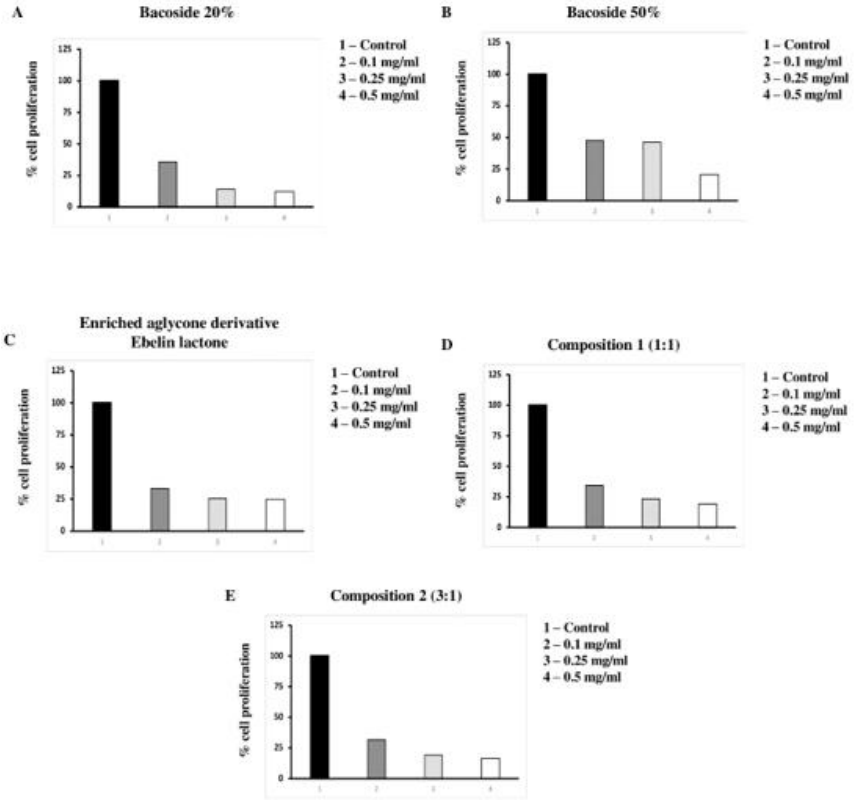

Fig. 7. Graph illustrating the percentage inhibition of proliferation of HCT116 cells

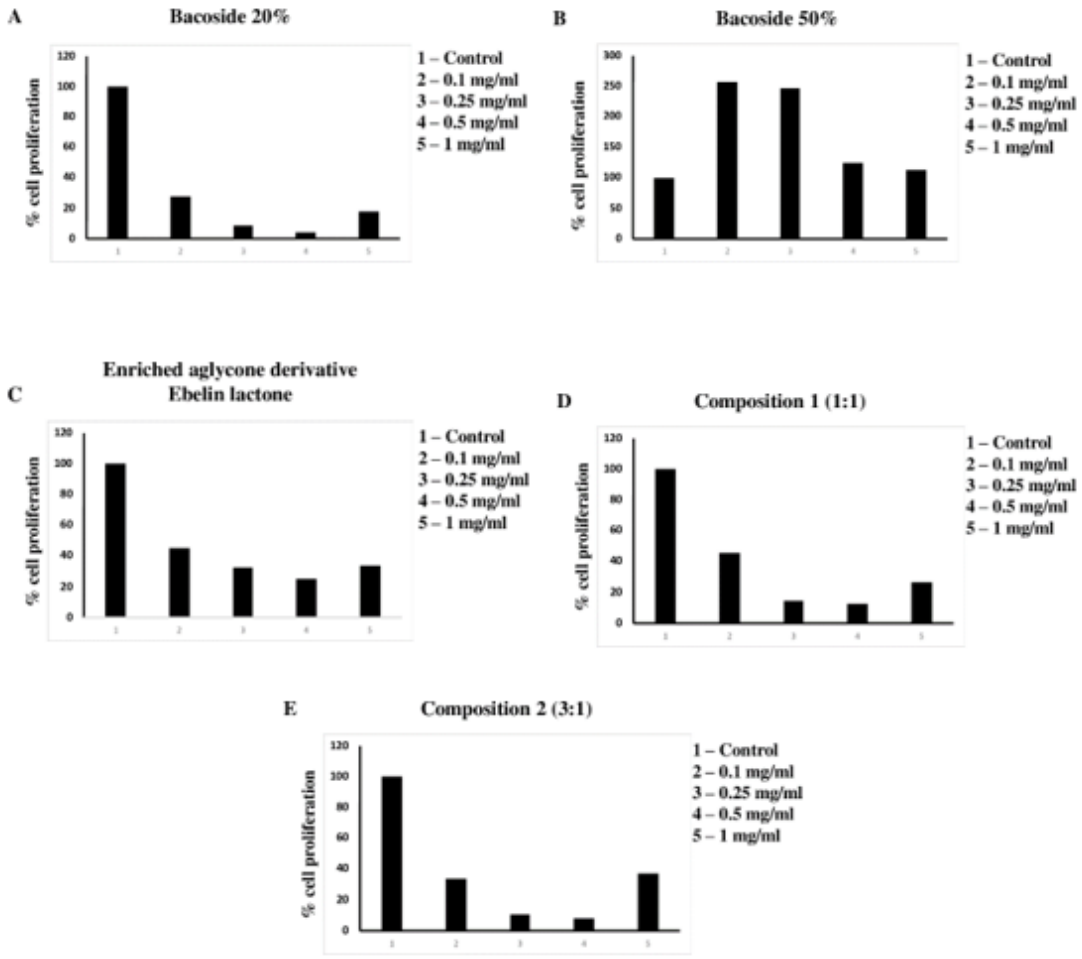

Fig. 8. Graph illustrating the percentage inhibition of proliferation of Hek293T cells

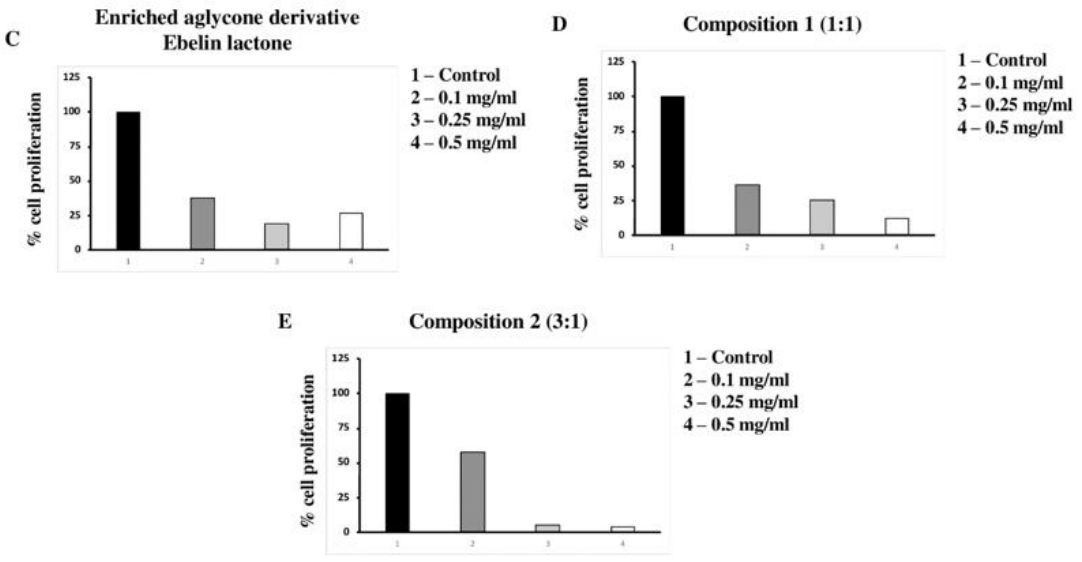

Fig. 9. Graph illustrating the percentage inhibition of proliferation of HFF-1 cells

4. CONCLUSION

Our findings contribute to the better scientific evidence demonstrating the bioactivity of *Bacopa* Composition 1 (1:1) which is a mixture of Bacosides with a high concentration of bacoside A3, jujubogenin, and an aglycone derivative, Ebelin lactone. In comparison to the parent Bacosides, our study is unique in the aspect that it focuses on making *B. monnieri* more accessible so that it may cross the blood-brain barrier (BBB) and be absorbed more readily from the intestines. Furthermore, *Bacopa* Composition 1 (1:1) has the additional benefit that the aglycone derivative components themselves are bioactive, as they have high free radical scavenging activity, acetylcholinesterase inhibition activity, anti-inflammatory activity, high absorption, and no toxicity. Thus, it synergistically enhances the bioactivity of Bacosides from *B. monnieri.*

The findings of such research will provide a better indication of *B. monnieri*'s potential for treating Alzheimer's disease. Furthermore, larger, longer-term trials comparing *B. monnieri* to currently available conventional medications are needed to evaluate whether *B. monnieri* is a viable alternative therapy in the treatment of various human illnesses.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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