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FORMULATION AND CHARACTERIZATION OF NOVEL MICROEMULSION OF *PHYLLANTHUS AMARUS* HAVING ANTI-HYPERALGESIC POTENTIAL

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ABSTRACT

Phyllanthus amarus is has been reported to possess wide range of pharmacological and therapeutic activities. The present study discloses a simple and improved microemulsion formulation system to enhance solublity and stability of standardised aqueous extract of *Phyllanthus amarus* [PAAE]. The solublity of *Phyllanthus amarus* aqueous extract in different oils, surfactant and cosurfactant was determined to find the optimal components for microemulsion. Rose oil was selected as oil phase, while tween 80 and PEG 400 were selected as surfactant and cosurfactant respectively. Based on higher solublity values Pseudo-ternary phase diagram was constructed in order to find the monodisperse region. The prepared microemulsion was evaluated for pH, viscosity, refractive index globular size, zeta potential, poly dispersity index, drug entrapment, percentage drug loading. The formulated microemulsion shows single phase distribution with excellent stability suitable for producing pharmacological activity by various routes of administration. The validated preclinical acute pain model was used to asses the Anti-hyperalgesic activity of the PAAE microemulsion viz. Carrageenan induced inflammatory hyperalgesia. The results of the present study indicate that the formulated microemulsion can alleviate muscle mediated pain and strongly suggest that this formulation can be useful in treatment of acute musculoskeletal hyperalgesia.

Keywords: *Phyllanthus amarus*, microemulsion, Anti-hyperalgesic

1. INTRODUCTION

Microemulsions are thermodynamically stable system in which two immiscible liquid solvents are mixed to make single phase by the addition of surfactant and co surfactant **[1-3]**. They have been used for delivery of both water soluble drugs and lipid soluble drugs. Micro emulsions can be utilized as drug delivery system for phytochemical compounds as it is thermo-dynamically stable, easy to formulate, have good appearance, and have large surface area with very small particle size in micrometer range **[1-3]**. The less solubility is the major barrier in the drug delivery of various phytoconstituents. Micro emulsions are the better drug delivery system for poor soluble phytoconstituents **[1-3]**. The small droplet size of microemulsions has the property of adhering to biological membranes and to transport these phyto-contituents in more controlled manner. Using microemulsion as drug delivery vehicles, lipophilic components from different phytochemical constituents can be co-solubilized to get synergistic effect for various therapeutic purposes. Micro emulsions have the ability to optimize the solubility, stability and permeability of phytochemical constituents **[1-3]**.

Phyllanthus amarus is has been reported to possess wide range of pharmacological and therapeutic activities **[4-6]**. The present study discloses a simple and improved microemulsion formulation system to enhance solubility and stability of standardized aqueous extract of *Phyllanthus amarus* [PAAE]. Micro-emulsion of aqueous extract of *P. amarus* was prepared by selecting appropriate solubility in oils and cosurfactant was selected. Ternary phase diagram was drawn to select appropriate ratio. The micro-emulsion was characterized by different tests SEM, zeta potential, particle size analysis, viscosity, Refractive index, pH and drug entrapment efficiency. The acute mechanical hypersensitivity of standardized aqueous extract of *P. amarus* micro-emulsion was investigated in Carrageenan induced inflammatory hyperalgesia as per previously reported procedures.

2. MATERIAL AND METHODS

The tween 80, tween 20, PEG 400,PEG 200 propylene glycol,carbitol were purchsed from Niram chemicals mumbai.Labrafil and Labrasol were purchased from sigma aldrich Germany.Various oils were purchased from local suppliers.The ethanol were purchased from Rajarambapu sahakari sugar factory LTD.

2.1 Excipient screening for the preparation of final optimized emulsion

Oil, surfactant and co surfactant were used as excipient and their selection was based

on solubility *Phyllanthus amarus* aqueous extract (PAAE) leading to a stable emulsion. Olive oil, almond oil, castor oil, clove oil. Coconut oil, rose oil, Arachis oil were selected as oil. Tween 20, Tween 80, PEG 400, PEG 200, propylene glycol, carbitol and ethanol were selected as surfactants and cosurfactant. Concisely to 1.0 ml oil or surfactant was added a maximum amount of PAAE. After that the tube was vortexed (72 .0 hour, 25.0 ^{0}C 1^0 C) using Remi CM-101 cyclomixer. A maximum amount of PAAE was removed by centrifugation (Remi centrifuge) of the mixture for (10.0 minutes rcf) followed by 72.0 hours.10.0 microlitres of supernant were transferred to fresh tube and volume and volume was made up to 1.0 ml with ethanol. The mixture was filtered first with syringe filter (0.22 micrometer) followed by vortexing. The correct dilution for absorbance at 285 nm to plot a calibration curve, followed by an unidentified amount of PAAE dissolved in dissolved in definite amount of oil or surfactant.

2.2. Construction of Pseudo ternary phase diagram

Pseudo ternary phase diagram was constructed to find the mono phase emulsion region. Phase diagram was constructed using 1:1 weight ratio of S/Co. Oil and S/Co$_{mix}$ ratios were mixed each other carefully at various weight ratios of 1.0: 9.0, 1.5; 8.5, 2.0: 8.0, 2.5: 8.5, 3.0: 7.0, 3.5: 6.5, 4.0: 6.0, 4.5: 5.5, 5.0: 5.0, 6.0: 4.0, 7.0: 3.0, 8.0: 2.0, 9.0: 1.0 and 1.5:7.0 (w/w) in 14 different glass tubes. Tubes containing the mixture solution were homogenously mixed for 4 min by vortex mixer (Thermo scientific) at room temperature. Water solution was added in small quantity of 50 µl. After addition mixtures were vortexed for 4 min to completely mix the sample and next addition was done after every 15 min of vortexing. After mixing, samples were visually observed against the dark background.

2.3 Preparation and optimization emulsion

PAAE was dissolved in oil and Smix in glass vial and a mixture was obtained which is micro titrated with milli-Q-water to achieve a coarse and stable emulsion. A high energy Ultrasonication technique was used in order to reduce the globule size of emulsion and to obtain stable emulsion. A 20 kHZ ultrasonic processor (Labman scientific instrument) was used for sonication. The amplitude of probe sonication was 37 % and temperature was 28.The sonication time was 45 minutes.

2.4 Characterization of emulsion

2.4.1 Droplet size (DS) and PDI measurements

A Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), utilizing DLS (dynamic light scattering) at 25 ^{0} C, was used to measure the droplet size and polydispersity index.

2.4.2 Particle surface charge (zeta potential) measurements

The Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK),maintained at 1.33 and 0.89 cp for refractive index and viscosity respectively in order to mimic the value of pure water, was applied to measure the zeta potential through electrophoretic mobility measurements. Immediately after the DS measurements, the potential was measured using the same cuvette with three successive readings for each sample, and the mean value and standard were calculated.

2.4.3 Determination of viscosity, Refractive index and pH

Viscosity of emulsion was determined by using Brookfield viscometer at 30 rpm rotation speed and by using spindle number. The refractive index is determined by using Abbes rerfractometer and pH of formulation was determined by using digital pH meter of equiptronics (EQ-615).

2.4.4 Entrapment efficiency and drug loading ratio of PAAE

Entrapment efficiency and drug loading ratio of EPE nanoemulsion were determined using a centrifugal filtration device (Microcon_ Millipore, Billerica, MA) with a 100 kDa molecular weight cut-off filter. Two hundred mL of EPE nanoemulsion were added to the sample reservoir of the Microcon system and then centrifuged at 1500_g at 4 _C for 45 min to separate the entrapped and untrapped components. The entrapped component in sample reservoir was washed twice with 200 ml of deionized water and the whole filtrate was collected and referred to as the untrapped component, which was evaporated and dissolved with 200 mL of DMSO. The entrapment efficiency (%EE) and drug loading ratio (DL ratio) were calculated using the following equations,

$$\%\ \text{entrapment efficiency} = \frac{\text{Entrapped phyllanthus amarus aqueous extract (mg)}}{\text{Initial extracts taken for formulation (mg)}} \times 100$$

$$\% \text{ drug loading ratio} = \frac{\text{Entrapped phyllanthus amarus aqueous extract (mg)}}{\text{Quantity of rose oil used in formulation (ml)}} \times 100$$

2.5 HPTLC analysis

Advanced phytochemical investigations were performed on HPTLC for identification and characterization of bioactive extracts, with the help and supervision of experts from Anchrom lab Mumbai. The details of the instrumentation parameters and procedure for finger print analysis is outlined as follows-

2.5.1. Instrumentation and chromatographic conditions utilized for HPTLC analysis

Instrument-CAMAG Linomat 5 "Linomat5_080222" S/N 080222 (1.00.12)

Apparatus-

(**a**) Spotting device.—Linomat V Automatic Sample Spotter (Camag, Muttenz, Switzerland).

(**b**) Syringe.—100 µl (Hamilton, Bonaduz, Switzerland).

(**c**) TLC chamber.—Glass twin-trough chamber (20 x10 x 4 cm; Camag).

(**d**) Densitometer.—TLC Scanner 3 linked to winCATS software (Camag).

(**e**) HPTLC plates.—20 x 10 cm, 0.2 mm layer thickness, precoated with silica gel 60 F254, Cat. No. 1.05548, E. Merck KgaA, Darmstadt, Germany.

Linomat 5 application parameters

Spray gas: Inert gas; Sample solvent type: Methanol; Dosage speed: 150 nl/s; Predosage volume: 0.2 ul; Syringe size: 100 µl; Number of tracks: 4- 8; Application position: 8.0 mm; Band length: 8.0 mm; Solvent front position: 80.0 mm.

2.5.2 Preparation of *Phyllanthus amarus* Sample Solutions [7-8]

Dried powdered extracts of *P. amarus (*200 mg) was re-extracted exhaustively with methanol using a sonicator for 1 h on a water bath. The methanol soluble portion was filtered and used for the further HPTLC analysis. The stock solution of the sample, having concentration of 0.4 mg/ml (0.4 µg/µl) was prepared.

2.5.3. Mobile Phases for General Finger Print Analysis [7-8]

Various mobile phases were tried such as, Toluene: Ethyl acetate (80:25); toluene: ethyl acetate: formic acid (60:20:20) for development of common chromatograms. But of the various mobile phases tried, Toluene: Chloroform: Ethanol (4:4:1, v/v) gave the best resolution for development of common chromatogram for the analysis of

the components of the PAAE under study from each other.

2.5.4. Mobile phases for detection of various Lignans class (Markers Phyllanthin and Hpophyllanthin) of compounds

- Chloroform: Methanol: Water (7:3:0.4, v/v) and the derivatization was carried with the help of Vanillin sulphuric acid.

2.5.5. General Finger Print Analysis [7-8]

HPTLC aluminum plates pre-coated with silica gel were used as the stationary phase. The plates were not pre-washed with any solvent prior to chromatography. The samples were spotted in the form of bands, with the help of a Camag 100 micro liter syringe using a Camag Linomat V (Switzerland) sample applicator. A constant application rate of 150 nL/s was employed. The slit dimension was kept at 6 mm × 0.45 mm, with a scanning speed of 20 mm/second, and a data resolution of 100 µm/step was employed.

The composition of the mobile phase was toluene: chloroform: ethanol (4:4:1). The linear ascending development was carried out in a twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 minutes at room temperature (25 ± 2°C). The length of the chromatogram run was 80 mm. Subsequently, the plate was allowed to dry at room temperature. The separated bands on the HPTLC plates were scanned over the wavelength of 200 – 540 nm. The source of radiation utilized was the deuterium illumination (D2 lamp) for 254nm, Mercury (Hg) for 366nm and for 540 nm. The images were captured on Camag reprostar 3 with win-CATS software 4.05.

2.6. Carrageenan induced inflammatory hyperalgesic model of acute pain [9]

Unilateral injection of carrageenan into the gastrocnemius muscle produces acute bilateral mechanical hyperalgesia. This model is possibly maintained by spinal or supraspinal neuronal mechanisms and has been suggested to have greater face validity to pain of muscoskeletal origin in humans.

2.6.1 Induction of carrageenan acute inflammatory muscle hyperalgesia.

Acute inflammation was induced by injecting 100 µL of freshly prepared solution of 3% carrageenan in normal saline to the left gastrocnemius muscle belly of rats, under light ether anesthesia. After 24 h intramuscular injection of carrageenan, spontaneous pain behavioral signs were observed in animals. Paw withdrawal latencies (PWLs) to mechanical stimuli were recorded in all groups; after injection of carrageenan.

2.6.2 Experimental protocol (dosages of PAAE and standard drug).

Inflammatory muscle pain was induced to all the animals as per the procedure described earlier. To investigate the effects of treatment on mechanical hypersensitivity, PAAE was administered to animals intraperitoneally after induction of pain. PAAE was administered to the rats. Standard drug used for comparison was Aceclofenac (preferential selective COX-2 inhibitor) given in a dose of 10 mg/kg, intraperitoneally. The evaluation of nociceptive responses was performed. Inflammatory control group (hyperalgesic rats) treated with vehicle dimethyl sulfoxide 0.2mL intraperitoneally was used for simultaneous comparative assessments with aceclofenac and PAAE treated groups. A parallel group of healthy rats (normal control) was kept to assess the level of muscle inflammation, and changes in prostaglandin E2 concentration. Animals were tested for PWLs to mechanical stimuli before carrageenan injection and consecutively there after till end of the study.

2.6.3 Behavioral testing for evaluation of mechanical hyperalgesia.

For testing of mechanical hyperalgesia or allodynia, the animals were placed on an elevated metal grid allowing stimulation of the plantar surface of the paw, and the animals were allowed to adapt to their environment for 15 min. The presence of mechanical hyperalgesia was assessed using a series of von Frey nylon hairs or filament (2–20 g), which were applied in increasing force until the rat withdrew its hindpaw. Each hair was applied five times, and the threshold (g) was taken as the lowest force that caused at least three withdrawals out of the five consecutive stimuli. Von Frey nylon hairs were calibrated both prior to and throughout the time course of the entire study to ensure that consistent bending forces were routinely applied. Responses to mechanical stimuli were also measured before and post intramuscular injection of carrageenan till end of study.

2.6.4 Measurement of muscle circumference.

The circumference of inflamed and the non-inflamed gastrocnemius muscle was measured over the skin using a measuring tape to confirm the development of inflammation. The circumference was also measured in subsequent groups after drug treatments to assess the correlation between PWL and inflammation. Muscle circumference measurement was performed by wrapping apiece of cotton thread round the muscle of each rat and measuring the muscle circumference with the help of a meter ruler as described previously. Muscle

circumference was measured before induction of inflammation and after intramuscular injection of carrageenan.

2.6.5 Measurement of prostaglandin E-2. Half milliliter supernatant inflammatory immersion of the left muscle was added to 2mL Potassium hydroxide-methanol solution (0.5 mol/L) and kept in a water bath at 50 °C for isomerization for 20 min, and then methanol was added to a total capacity of 5mL and thoroughly mixed. After standing for 5 min, the absorbance was measured at 278nm using a ultraviolet spectrophotometer (Shimadzu 1800). The optical density value of per milliliter inflammatory exudates corresponds to indicate the prostaglandin E-2 (PGE2) content.

2.7. Molecular Docking Studies of PAAE [10]

The docking analysis of the target protein Prostaglandin with the marker phytochemical ligands (Phyllanthin and Hypophyllanthin) was performed using the VLife MDS software. Phyllanthin and Hypophyllanthin phyto constituents identified from phyllanthus species were selected for the present study. VLifeMDS provided a facility to dock different ligands in protein binding sites chosen by the user. VLifeMDS provided both rigid (no torsional flexibility for a protein as well as a ligand) and flexible (torsional flexibility to a ligand with a rigid protein) docking of the molecules. The target or receptor was either experimentally known or theoretically generated through knowledge-based protein modeling or homology modeling. The molecular docking tool has been developed to obtain a preferred geometry of interaction of ligand–receptor complexes having minimum interaction energy based on different scoring functions viz. only electrostatics, the sum of steric and electrostatic (parameters from the force field), and the dock score. This utility allowed us to screen a set of compounds for lead optimization. VLife MDS uses the genetic algorithm, Piecewise Linear Pair wise Potential (PLP) and Grid algorithms to minimize the interaction energy between the ligand and receptor protein.

The downloaded protein databank file of the target protein prostaglandin checked for any errors in the protein structure with the help of biopredicta tools. The target protein was checked for crisscross residues, a local geometry check, and a Ramachandran plot with the help of Biopredicta tools and coordinates. The structures of phytocompounds (ligands) were drawn in 2D and converted into an optimized 3D form before using VLifeMDS computational software. The phytochemical ligands were docked with the selected Prostaglandin receptor. The

protein-ligand interactions were observed during docking analysis, concentrating on the study of the docked poses which showed significant dock scores. The Pi stacking, H-bonding, and hydrophobic interactions of the ligands with receptor proteins were analyzed which revealed a novel set of information.

3. RESULT AND DISCUSSION

3.1 Pharmaceutical characterization of PAAE Microemulsion

The formulated emulsion of aqueous extract of *Phyllanthus amarus* is depicted in **Figure 1**.

3.1.1 Excipient screening for the preparation of final optimized emulsion

PAAE solubility was determined in various oils Olive oil, almond oil, castor oil, clove oil. Coconut oil, rose oil, Arachis oil as displayed in **Figure 2**. Finally, we selected rose oil that showed maximum solubility. Tween-80 with HLB value of 15.00 exhibited maximum solubility, followed by PEG-400. Based on these observations rose oil was selected as oil, Tween-80 as surfactant and PEG-400 as co surfactant. Solubility of PAAE in surfactants is shown in **Figure 3**. Solubility of PAAE in cosurfactants is shown in **Figure 4**. While **Figure 5**. Ternary phase diagram containing rose oil, tween 80 and distilled water.

3.1.2 Emulsion globule size

The particle size of emulsion was found 2116.6s nm (2.1166 micrometer) at scattering angle 173 at holder temperature 24.8 °C obtained on the basis of light scattering intensity. The distribution form (dispersity) was found monodisperse with count rate 1292 kCPS. The distribution form was narrow. The data is summarized in **Table 1**.

3.1.3 Polydispersity index

PDI size generally range in between 0.05-0.7**.**PDI values bigger than 0.7 indicate that the sample has a very broad particle size distribution and is probably not suitable to be analyzed by the dynamic light scattering (DLS) technique. The PDI of PAAE was determined by dynamic light scattering method by using Malvern instrument (Horiba scientific SZ-100). The PDI was found **0.202.** Which indicates stable and monomodal particle size distribution.

3.1.4 Viscosity

The viscosity of PAAE was found 19.41 CP determined by using Brookfield viscometer.

3.1.5 Refractive index

The refractive index of PAAE was found 1.6050 determined by Abbes refractometer.

3.1.6 pH

The pH of PAAE emulsion was found to be 7.74.

3.1.7 Entrapment efficiency- The freshly prepared PAAE emulsion was found 81.10

% which is sufficient to use formulations for evaluation of pharmacological activity.

3.1.8 Percentage drug loading- The % drug loading in the formulation was found to be 80 %.

3.1.9 Zeta Potential

The mean zeta potential of PAAE was found - 0.4 mv, (while mean Electrophoretic was recorded to be 0.00003) which indicates there is sufficient charge potential exist on the droplets of emulsion which will prevent aggregation of emulsion droplets which each other and provide sufficient stability to the formulation.

Graphical summarization of Zeta potential of PAAE emulsion is shown in **Figure 6**. Zeta potential characteristics of PAAE is summarized in **Table 2**.

3.2 HPTLC analysis of PAAE

3.2.1 HPTLC Finger Print analysis of PAAE

HPTLC fingerprint study demonstrates unique finger print pattern for the similar solvent system. The HPTLC plate pictures are depicted under specific heading of wavelength in the **Table 3**.

Chromatographic finger print analysis of phyllanthus extract taken at 254 nm wavelength shows Rf unique loci at 0.26. PAAE showed presences of 4 compounds. HPTLC finger print profile and 3d spectra of PAAE taken at 254 nm wavelength recorded. The details of Rf range, No of Peaks, No of Auto Generated tracks and Maximum height of peaks is summarized in **Table 3**. Chromatographic finger print analysis of phyllanthus extracts taken at 366 nm wavelength shows Rf unique loci at 0.68. PAAE showed presences of 11 compounds. The number of auto generated peaks for PAAE was 3. HPTLC finger print profile and 3d spectra of phyllanthus extracts taken at 366 nm are recorded in the **Figure 7**. Chromatographic finger print analysis of the derivatised PAAE taken at 366 nm wavelength shows Rf unique loci at 0.41. PAAE showed presences of 12 compounds. HPTLC finger print profile and 3d spectra of subsequent derivatization of PAAE taken at 366 nm wavelength. The maximum height peak of 407.6 was observed with PAAE extract. The number of auto generated peaks for were 12. Chromatographic finger print analysis of the PAAE taken at 540 nm wavelength shows Rf unique loci at 0.68. PAAE showed presence of 9 compounds.

3.2.2 Detection of Lignans in PAAE

For detection of Lignans mobile phase used was chloroform: methanol: water (7:3:0.4, v/v) and the derivatization was carried with the help of Vanillin sulphuric acid.

Chromatographic finger print analysis of the phyllanthus extracts taken at 254 nm wavelength for detection of lignans shows unique Rf loci at 0.61. PAAE showed presences of 11 compounds of lignan class. HPTLC finger print profile and 3d spectra for detection of lignans in phyllanthus extracts taken at 254 nm wavelength is depicted in **Figure 8**.

The number of auto generated peaks for PAAE 15. The Rf range for PAAE was 0.12 - 0.74. The details of Rf range, No of Peaks, No of Auto Generated tracks and Maximum height of peaks is summarized in **Table 4**.

3.2.3 HPTLC finger print matching of markers compounds

The identities of the bands of standard marker compounds like phyllanthin, and hypophyllanthin in the phyllanthus extracts were confirmed by overlaying their UV absorption spectra with those of the standards compounds from the data base of the Anchrom lab. As the quantitative estimation was not possible, however the overlaying their UV absorption spectra and identification of peaks are more than useful for identification of these marker compounds.

The HPTLC chromatogram of Phyllanthus extracts for detection of lignans with help of standard markers phyllanthin (P) and hypophyllanthin (H) using mobile phase composed of chloroform: methanol: water (7:3:0.4, v/v) for *P. amarus* aqueous extract are depicted in **Figure 10**.

3.4 Effect of PAAE on Carrageenan induced Inflammatory Hyperalgesia.

3.4.1. Effect of carrageenan injection on the gastrocnemius muscle inflammation

Injection of 3% carrageenan into the left gastrocnemius muscle produced inflammation of the muscle at 24 h and was still present after 2 days in acute model. The inflammation was accompanied by a significant reduction in paw withdrawal latency to mechanical stimuli.

3.4.2. Spontaneous pain behavior

The Spontaneous pain behavior signs were observed in animals such as guarding the injected paw and weight bearing on the contra lateral paw during the period of experimentation.

3.4.3. Effect of PAAE on mechanical hyperalgesia

Basal PWLs to mechanical stimuli for all the groups in the experiment after carrageenan injection reduced significantly. Effects on Paw withdrawal latencies of mechanical hyperalgesia after carrageenan into the muscle on the ipsilateral and contralateral paws. A maximum decrease in mechanical response threshold was observed in the ipsilateral as well as contralateral paws in animals treated with

vehicle alone. The intraperitoneal administration of PAAE caused a rapid reduction in mechanical hyperalgesia (allodynia) returning it to near normal values within 1 hour. They avoided the spontaneous pain behavior and an exaggerated response to mechanical stimuli. The PAAE at the studied dose (400 mg/kg) showed a significant reduction in mechanical hyperalgesia induced by carrageenan. The results show PAAE significantly attenuated mechanical hyperalgesia, $p<0.05$ versus inflammatory control in both paws. The effects of PAAE, aceclofenac, and vehicle (inflammatory control) administered post carrageenan injection on mechanical hyperalgesia are summarized in **Figure 7**. Data also revealed that treatment with PAAE inhibited mechanical hyperalgesia ($p<0.01$ versus inflammatory control) with similar efficacy to aceclofenac ($p<0.01$versus control).

3.4.4. Effects of PAAE on muscle inflammation

Carrageenan produced distinct muscle inflammation in the inflammatory control group indicating inflammatory response as compared with the normal control animals. While the consequent dosing of the PAAE showed marked inhibition of muscle inflammation as there was a significant decrease in circumference of muscle as compared with the inflammatory control **(see Figure 8)**. Effect of administration of PAAE, aceclofenac, or vehicle administered on muscle edema induced by carrageenan. Muscle diameter was measured only ipsilaterally. Each point represents the mean ± standard error of mean of muscle thickness/diameter (in centimeters) before carrageenan injection (baseline) or at the times after intramuscular injection of Carrageenan.

3.4.5. Effects on concentration of PGE2 level

Effect of administration of PAAE on PGE2 concentration in muscle exudates on after intramuscular injection of carrageenan in rats was noted. The treatment with PAAE significantly decreased PGE2 level in the edema exudates as compared with inflammatory control group. The inhibitory potency of PAAE treated groups was also better as compared with the control. A concentration response curve was observed after the treatment with PAAE on concentration of PGE2, as there was a gradual decrease in concentration of PGE2 with increase in dose of PAAE. PGE2 concentration was measured only ipsilateral carrageeenan injected muscle. The basal concentration of PGE2 (normal control animals without inflammation) was 0.186 ± 0.26 as compared with 8.736 ± 0.18 with the inflammatory control animals. PAAE

(400 mg/kg) and aceclofenac significantly ($p<0.01$) attenuated the PGE2 concentration in muscle exudates. The comparative details are summarized in **Figure 9**.

3.4.6 Antihyperalgesic effects of PAAE in carrageenan induced acute hyperalgesia

In the present study, we have examined the effects of systemic administration of the Phyllanthus extracts, on thermal and mechanical hyperalgesia evoked by intramuscular injection of carrageenan in model of muscle hyperalgesia. It is clear from the results of our study that PAAE can reverse the already established hyperalgesia. The results of our study suggest that muscle mediated inflammatory pain can be alleviated by PAAE and that once established, ongoing inflammation does not appear to contribute to the process of inflammatory hyperalgesia. It is important to mention that the antihyperalgesic effects of PAAE were not susceptible to tolerance, because PAAE maintains its efficacy when administered repeatedly by intraperitoneal route. The mechanical hyperalgesia produced in the present model are maintained by spinal or supraspinal neuronal mechanisms, as a result from series of central and peripheral changes occurring at the site of insult **[9]**.

Spinal COX-2 plays an important role in the maintenance of hyperalgesia induced by carrageenan **[11-12]**. After inflammation, allodynia and hyperalgesia occur, generally because of an increase in PGE2 level in the inflamed tissue and in the spinal cord that can be associated with induction and activation of COX-2 **[11-12]**. The COX-2 enzyme is the major source of PGE2 in many inflammatory pain models, and almost all of the COX-2-selective inhibitors have shown potent antihyperalgesic activity **[11-12]**. The inhibition of PGE2 synthesis by nonsteroidal antiinflammatory drugs and COX-2 inhibitors contributes to their efficacy in treating the signs of acute inflammatory pain **[13-14]**. In the present study, there was a significant decrease in PGE2 level in the edema exudates for the *P. amarus* and aceclofenac treated groups as compared with the inflammatory control.

In the present study, it is clear from the phytochemical studies that the PAAE contains high amount of lignans, in good proportion. Lignans are the major constituents present in the Phyllanthus extract and are reported to possess analgesic, antioxidant, antiinflammatory, anti-arthritic, and immunomodulatory activity **[15-16]**. Purified lignans such as phyltetralin, nirtetralin, and niranthin isolated from Phyllanthus extracts have shown antiinflammatory actions in vivo and in vitro experiments **[14-17]**. The phytochemical finger print analysis of the

Phyllanthus extract clearly indicates that the observed antihyperalgesic activity in the studied extract can directly be assigned to presence of lignans and tannins **[18-19]**.

From the HPTLC chromatograms, we can interpret that PAAE contain considerable amount of lignans (phyllanthin and hypophyllanthin). Therefore, the presence of these compounds might be the ultimate cause for their bioactivity. Studies from our lab have demonstrated acute pain modulating potential of Phyllanthus species. The molecular docking analysis of phytocompounds from Phyllanthus species with inflammatory target involving enzyme like PGE synthase suggests that lignans have ability to interact with PGE target involved in inflammatory hyperalgesia and its modulation.

The results of the present study confirm the earlier findings that the lignans from the Phyllanthus species exhibit significant antihyperalgesic activity and are responsible for the observed pain modulating potential. The observed anti-hyperalgesic and antiinflammatory effects of PAAE in carrageenan induced pain model may be due to the presence of phyto-constituents like phyllanthin, hypo-phyllanthin, which offers a promising means for the treatment of inflammatory muscular pain.

3.5. Molecular docking (Inhibition of Prostaglandin Synthesis)

Prostaglandins (PGs) have numerous and diverse biological effects on a variety of physiological and pathological events, such as the contraction of smooth muscle, inflammation, and blood clotting. Out of the other types of prostaglandins, the PGE2 type plays a pivotal role in inflammatory hyperalgesia. The crystal structure of N-terminal truncated mPGES-2 complexed with indomethacin, a significant non-steroidal anti-inflammatory drug, has been proposed. The crystal structure indicates that indomethacin inhibits both PGH2 synthesis and PGE2 synthesis **[12]**. Evidence supporting the importance of PGE2 in the feedback loop comes from a previous study describing the induction of COX-2 expression by prostaglandins in human and mouse cell lines. The inhibition of PGE2 synthesis by NSAIDs and COX-2 inhibitors contributes to their efficacy in treating the signs of inflammatory pain **[11-13]**. In our studies in the model of carrageenan hyperalgesia, prostaglandins play an important role in evoking pain and hyperalgesia which the PAAE have shown good effects in the inhibition of hyperalgesia. In the present docking analysis, the lignans hypophyllanthin showed good docking of microsomal prostaglandin E synthase type 2 (mPGES-

2) in the present study. The results of the docking analysis and the interactions with the selected receptor proteins are summarized in the **Figure 12** and **Table 5**.

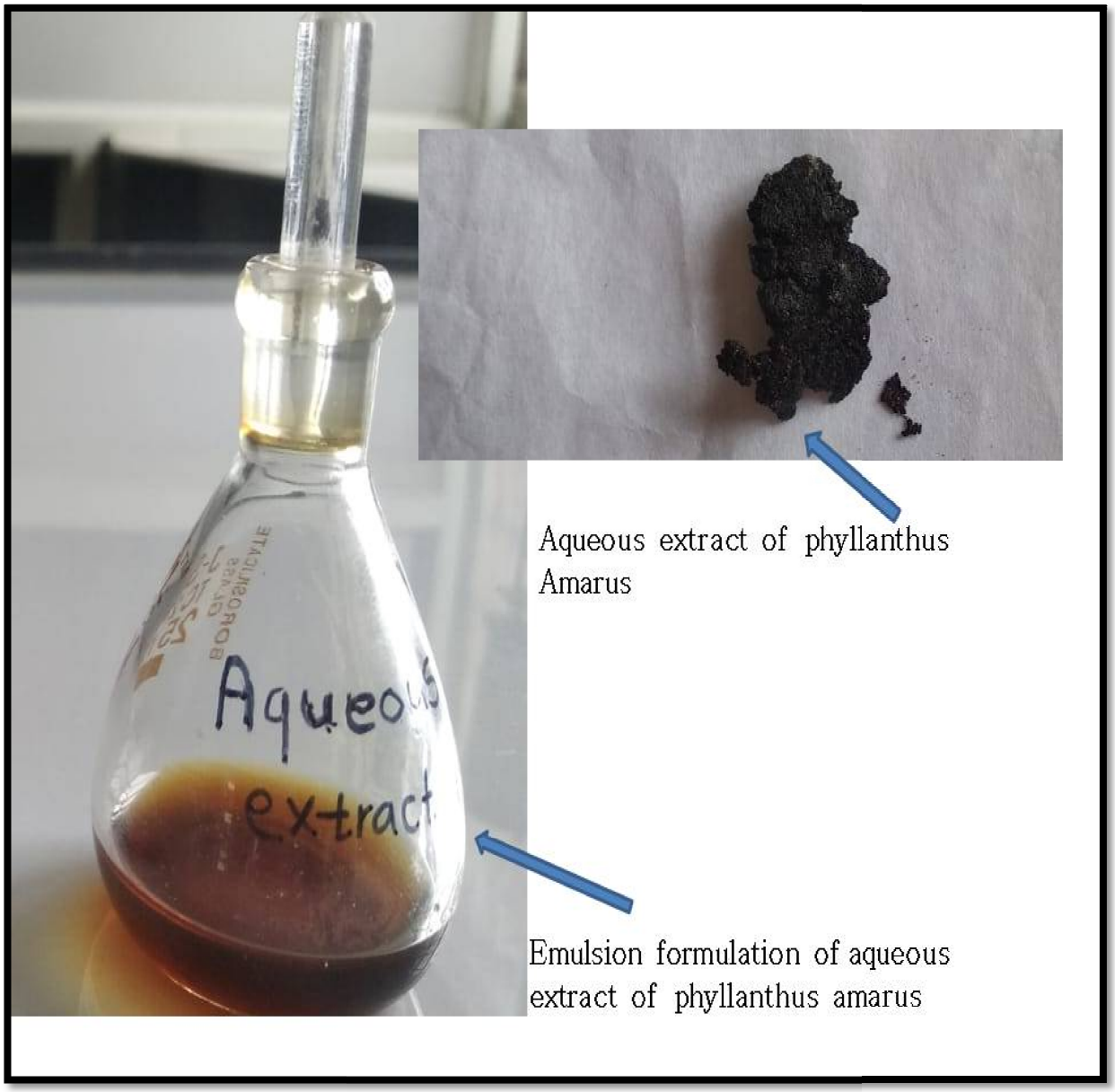

Figure 1: Emulsion of aqueous extract of *Phyllanthus amarus*

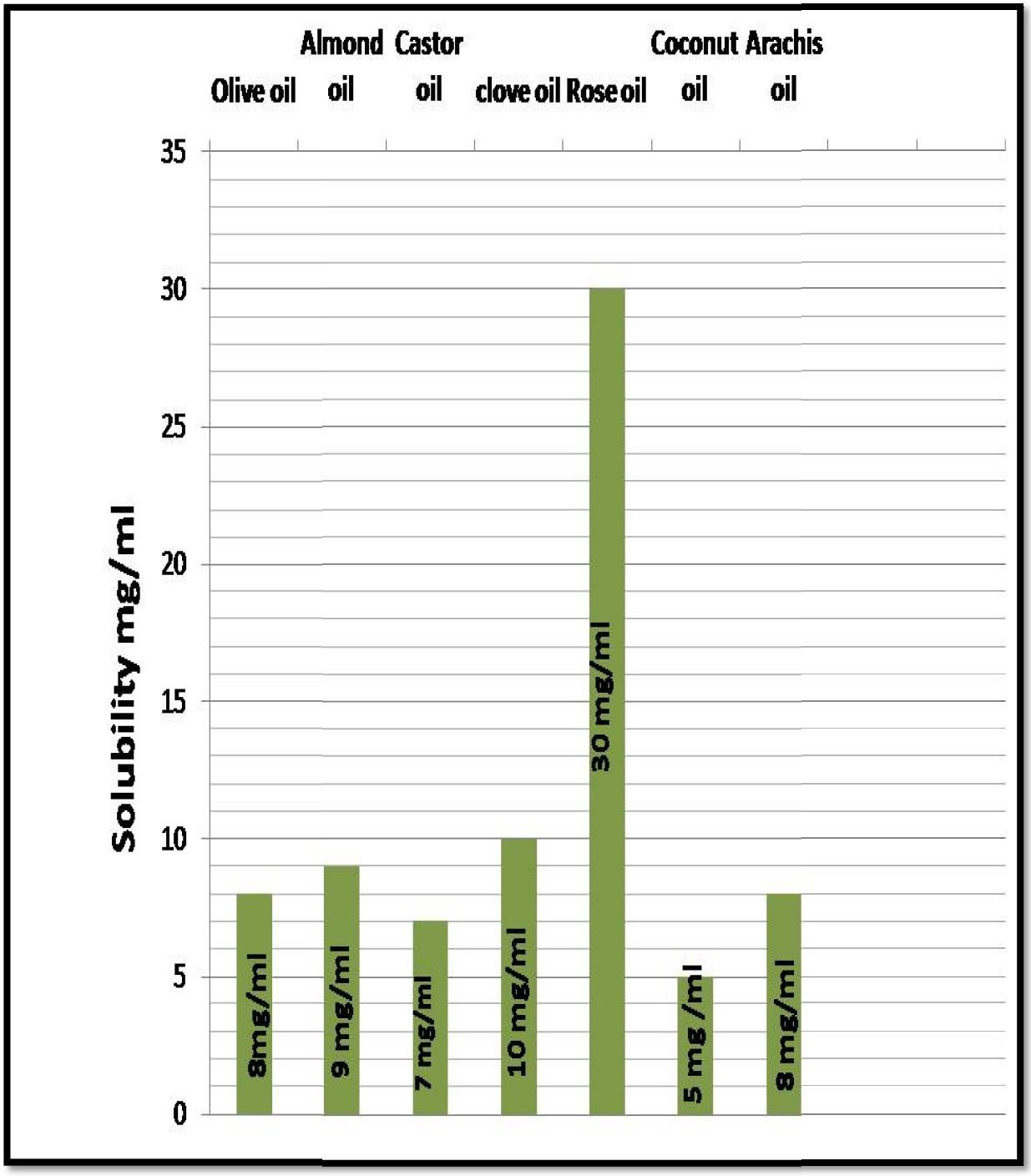

Figure 2: Solubility of PAAE in different oils

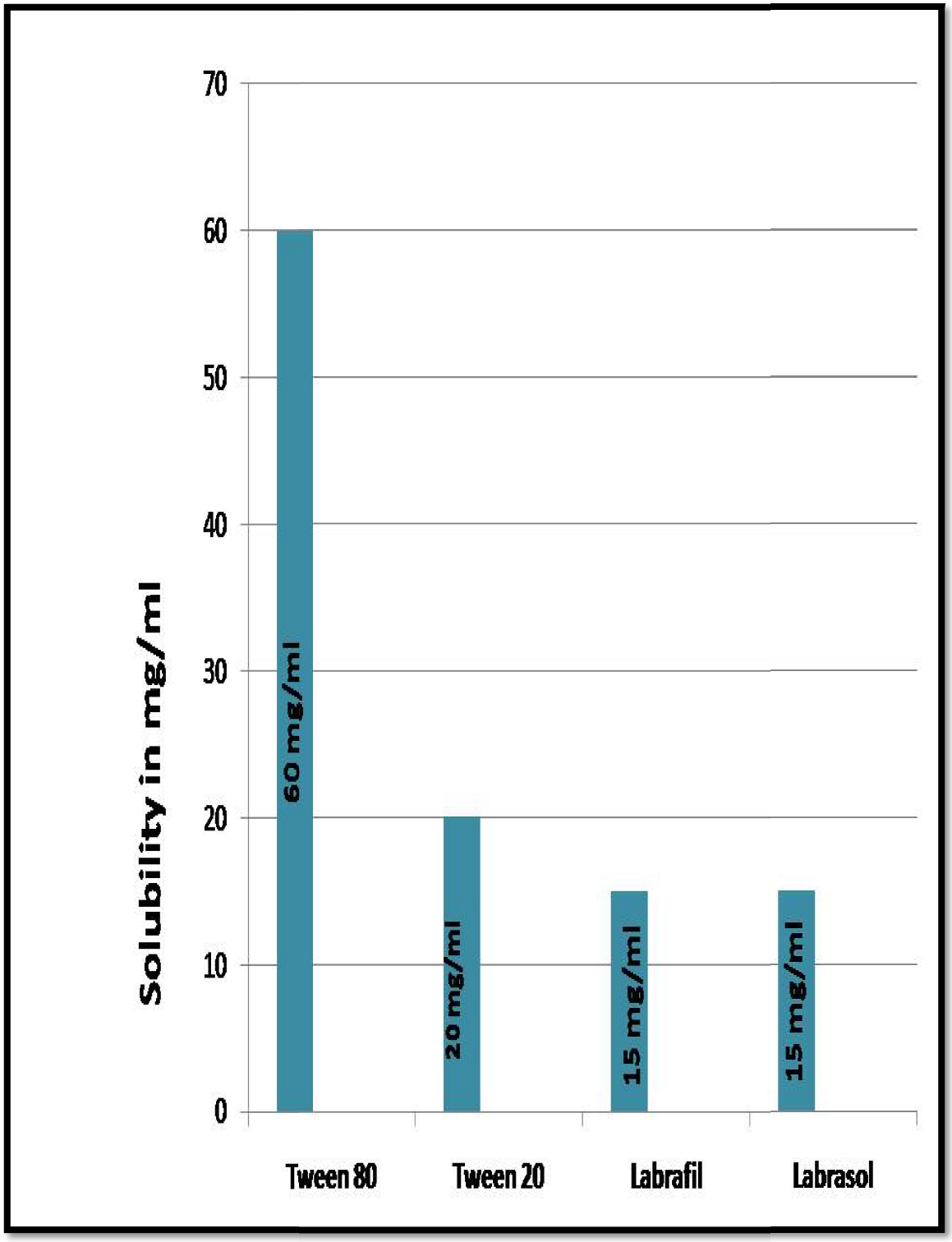

Figure 3: Solubility of PAAE in surfactants

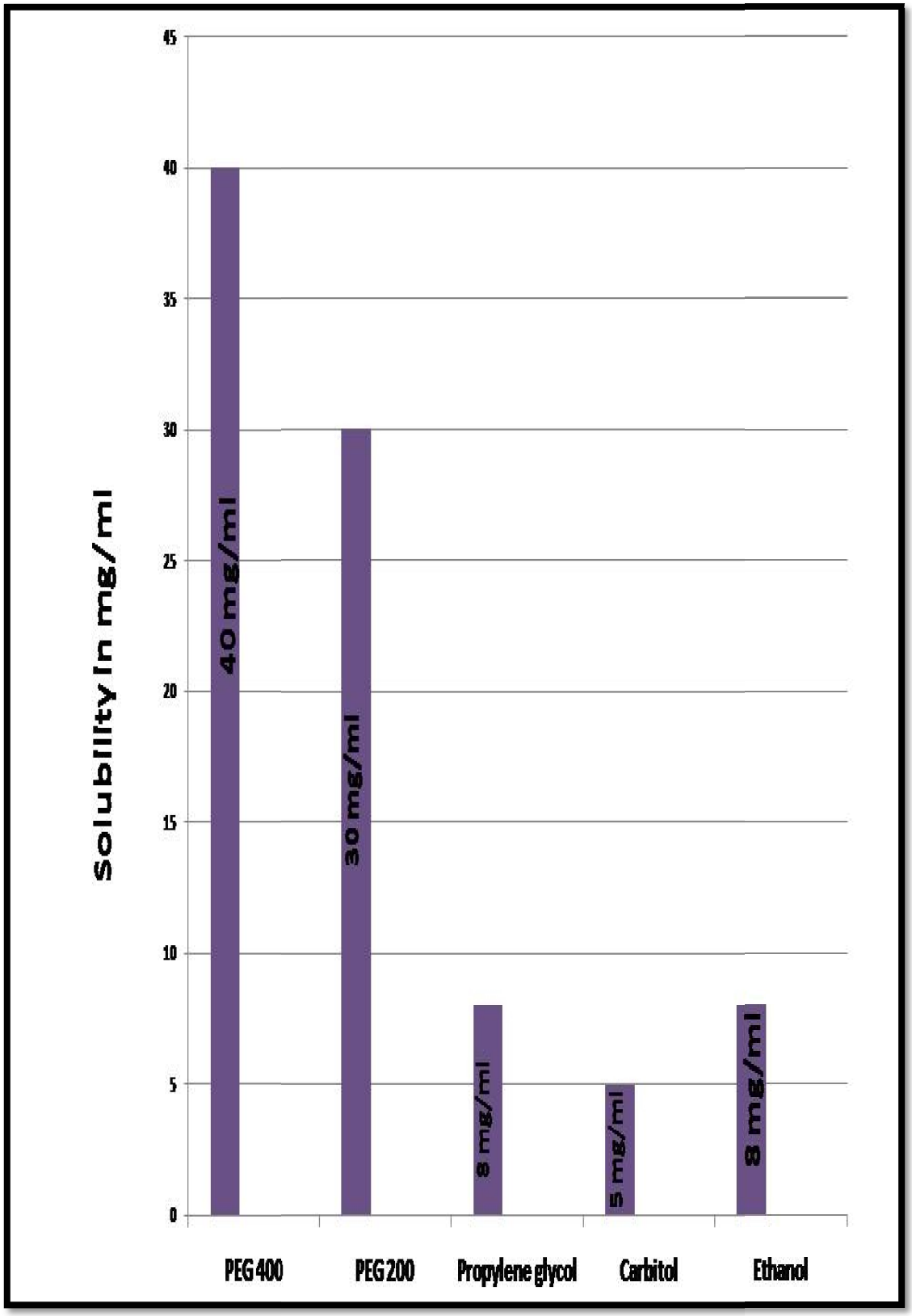

Figure 4: Solubility of PAAE in cosurfactants

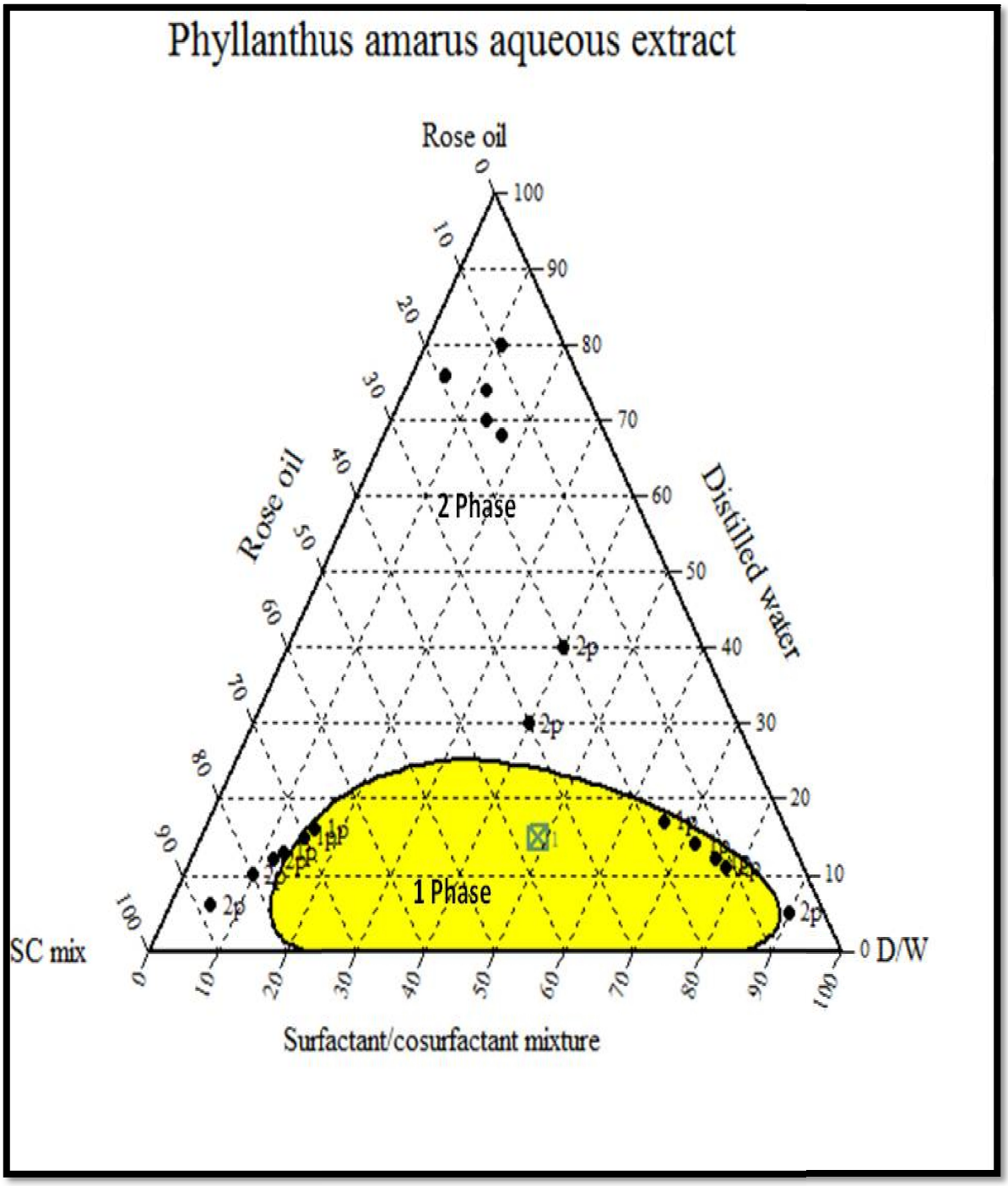

Figure 5: Ternary phase diagram containing rose oil, tween 80 and distilled water

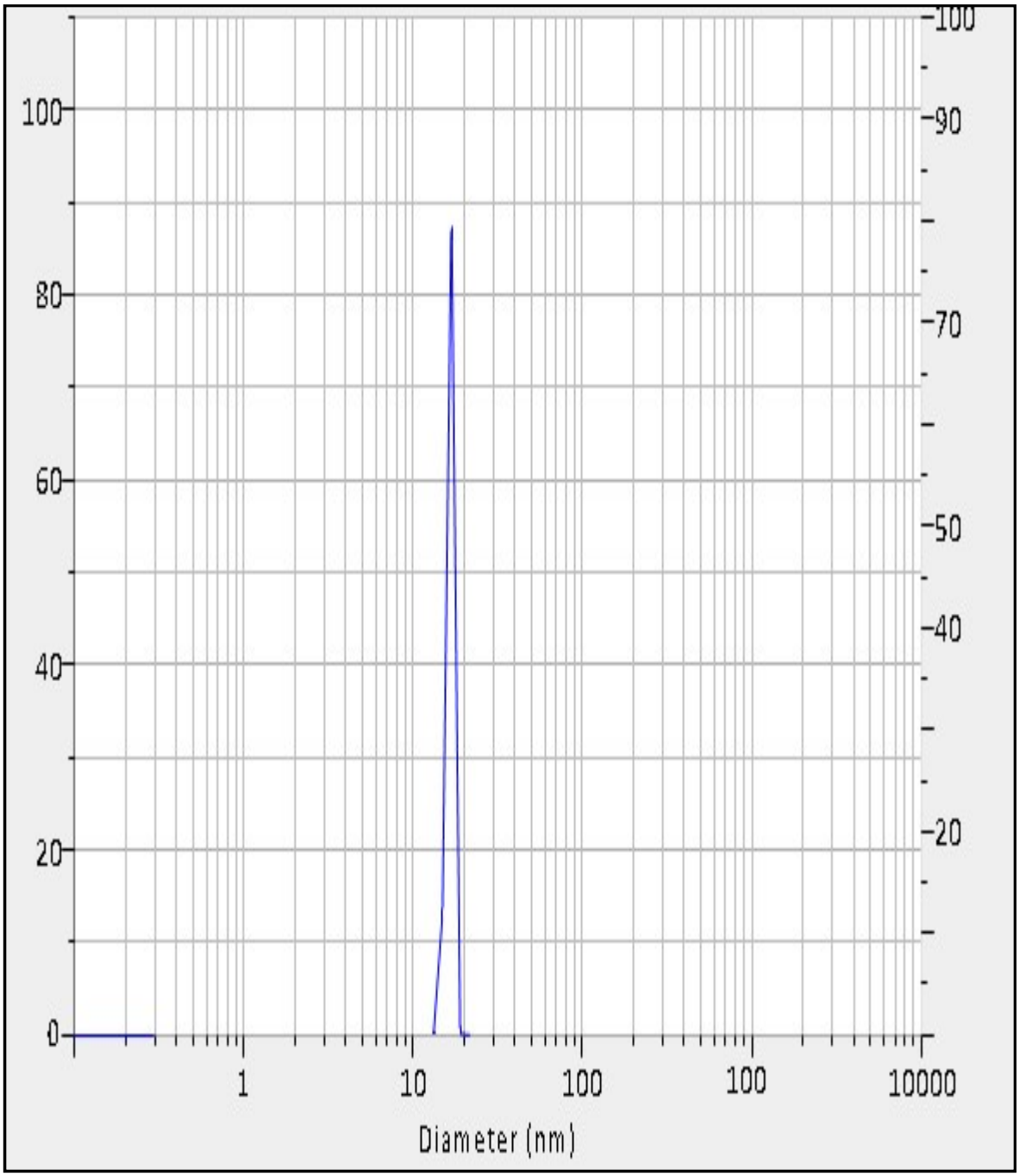

Figure 6: Zeta potential of PAAE emulsion

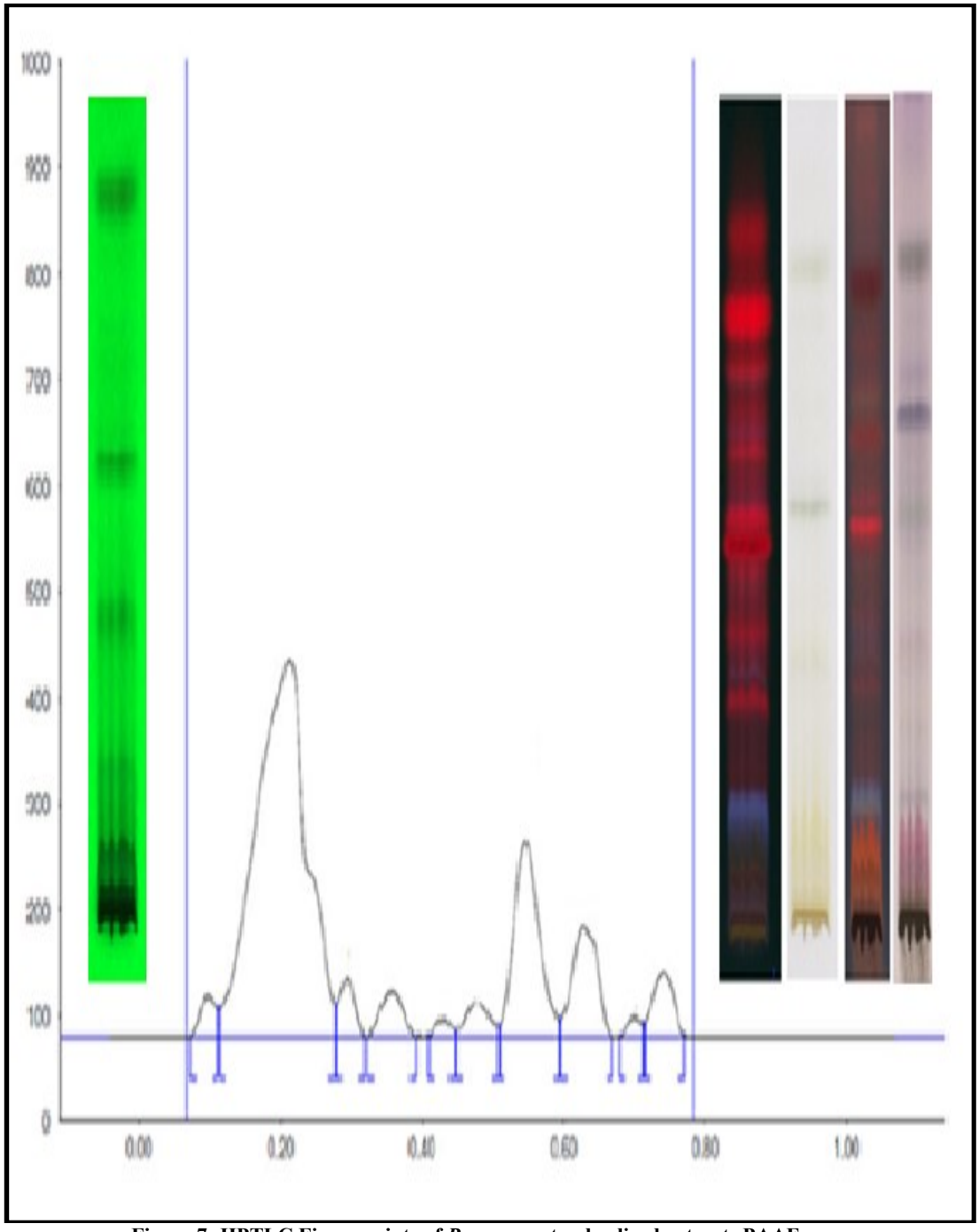

Figure 7: HPTLC Finger prints of *P. amarus* standardized extracts PAAE

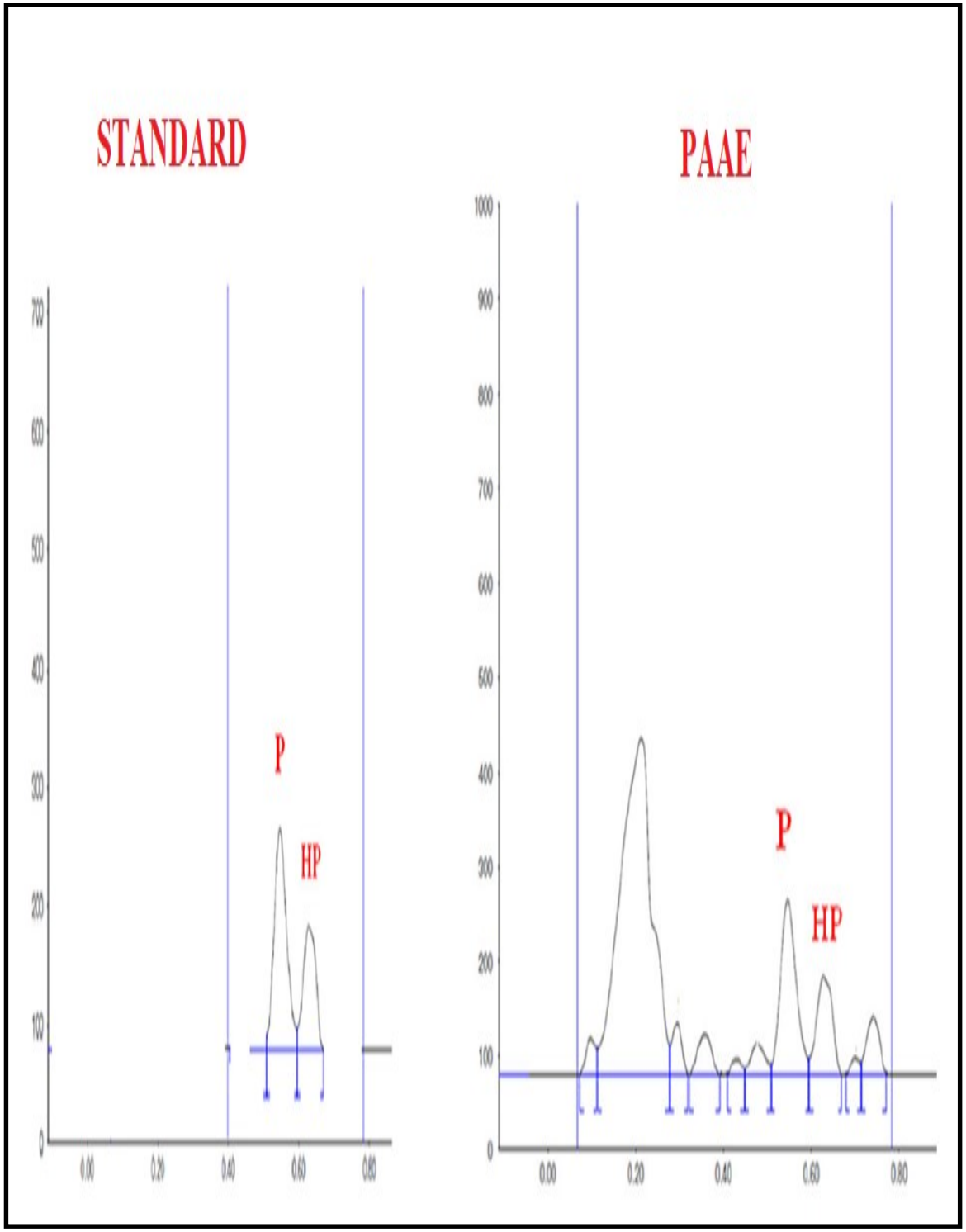

Figure 8: HPTLC finger print profile of PAAE for marker compounds (P- Phyllanthin, HP- Hypophyllanthin)

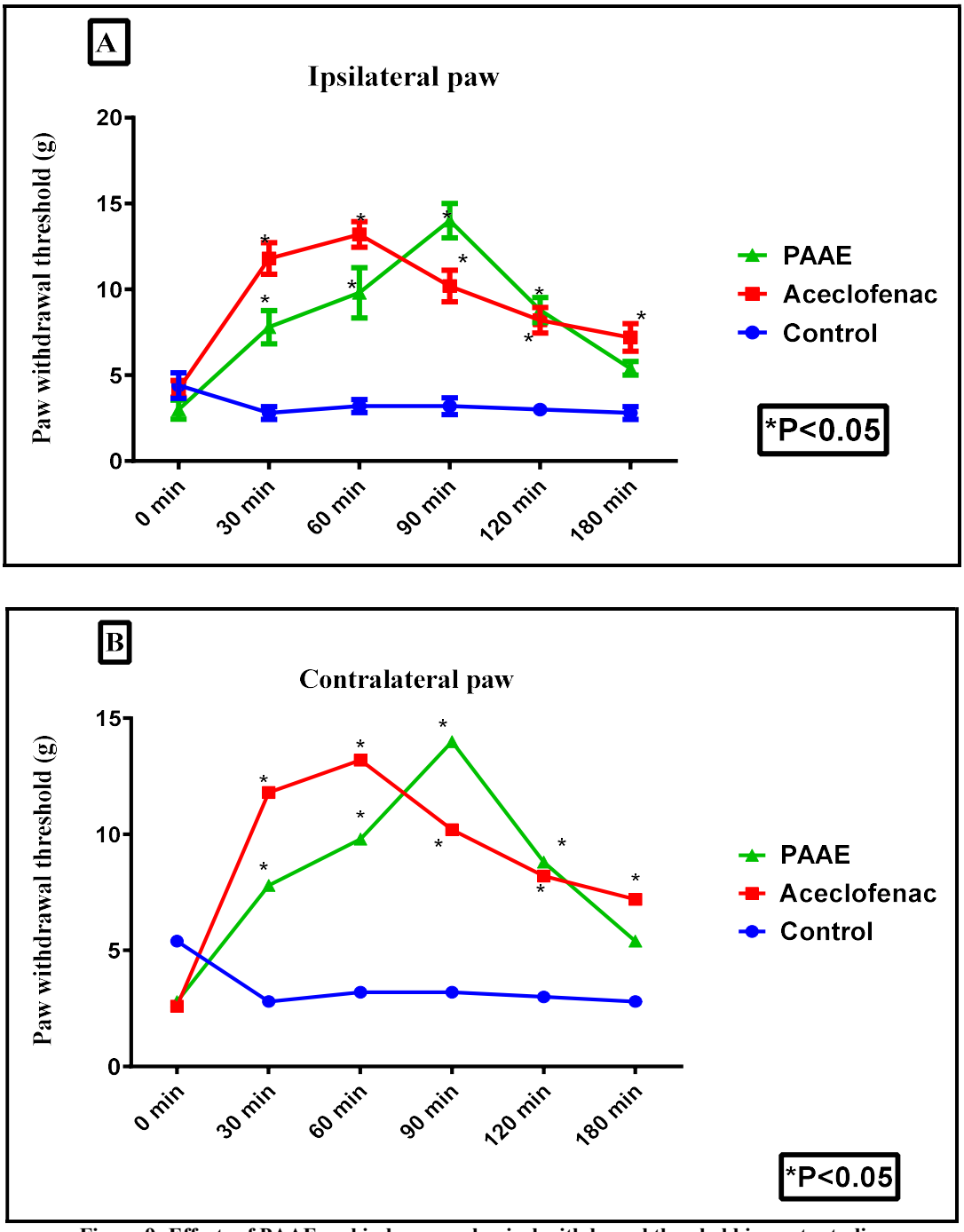

Figure 9: Effects of PAAE on hindpaw mechanical withdrawal threshold in acute studies

PAAE was injected immediately after the baseline responses had been obtained. PAAE induced an increase in ipsilateral (A) and contralateral (B) paw withdrawal threshold, which remained significantly different from baseline and vehicle treated control for up to 180 min after injection of PAAE and Aceclofenac. Data are presented as mean ± SEM,*P < 0.05 vs. corresponding vehicle time points

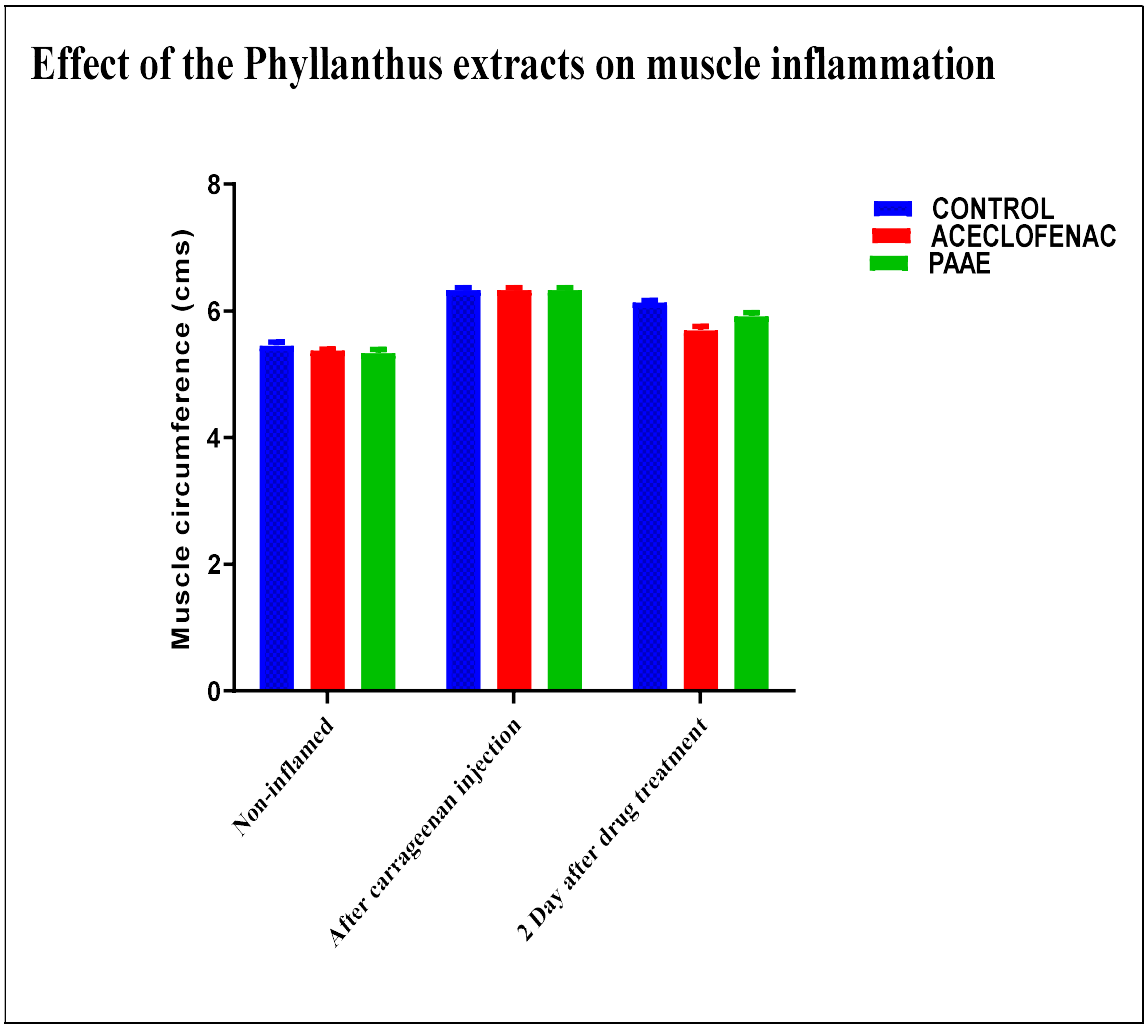

Figure 10: Effect of PAAE on muscle inflammation in chronic inflammatory hyperalgesia

Effect of administration of Phyllanthus extracts, aceclofenac, or vehicle administered on muscle edema induced by carrageenan. Muscle diameter was measured only ipsilaterally. Each point represents the mean ± standard error of mean of muscle thickness/diameter (in centimeters) before carrageenan injection (baseline) or at the times (2nd day) after intramuscular injection of Carrageenan.

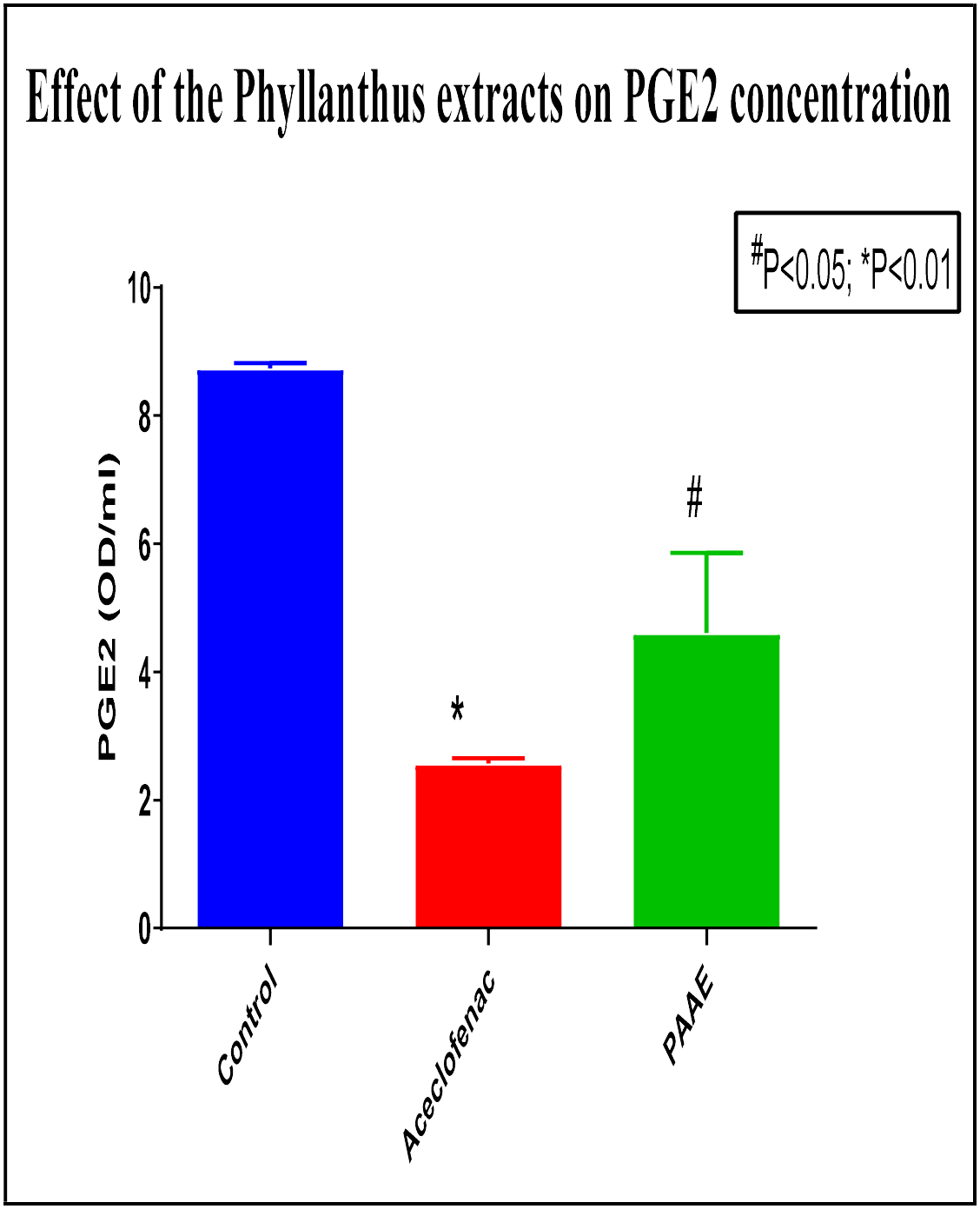

Figure 11: Effects of PAAE on PGE2 concentration in muscle exudates induced by Carrageenan in rats
Each bar represents the mean ± standard error of mean of the PGE2 concentration (in optical density/ml). Data were analyzed by one-way analysis of variance using Dunnett's multiple comparison test. p<0.05 was considered significant in comparison with inflammatory control

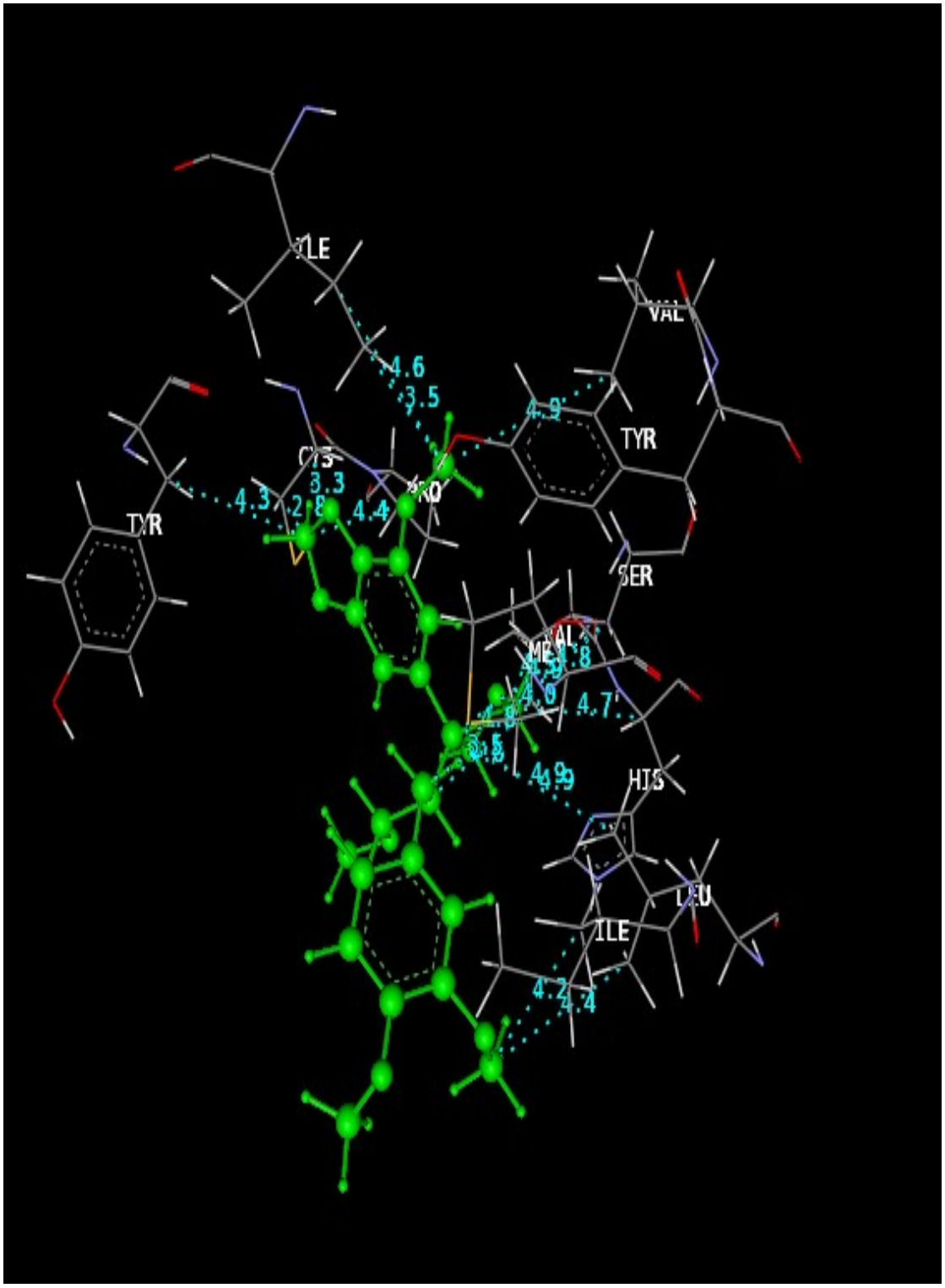

Figure 12: Best docking of poses of Hypophyllanthin with PGE

Table 1: Emulsion globule size of PAAE

Measurement Type	Particle Size
Sample Name	Aq extract
Scattering Angle	173
Temperature of the Holder	24.8 °C
Dispersion Medium Viscosity	0.898 mPa·s
Transmission Intensity before Meas.	10
Distribution Form	Narrow
Distribution Form (Dispersity)	Monodisperse
Representation of Result	Scattering Light Intensity
Count Rate	1292 kCPS

Table 2: Zeta potential characteristics of PAAE

Measurement Type	Zeta Potential
Sample Name	Aqueous extract
Temperature of the Holder	24.8 °C
Dispersion Medium Viscosity	0.898 mPa·s
Conductivity	0.083 mS/cm
Electrode Voltage	3.9 V

Table 3: Chromatographic finger print analysis of PAAE taken at various wavelengths

Name of extract	Parameters					
	Rf range	Rf of Unique loci	No of Peaks	No of Auto Generated tracks	Max Height	Area % range
PAAE at 254 nm	0.02 -0.40	0.25	4	4	92.6 - 225.0	11.52 - 50.89
PAAE at 366 nm	0.03 - 0.68	0.68	11	3	10.6- 431.3	0.15 - 26.72
Derivatised PAAE taken at 366 nm	0.04 - 0.72	0.41	12	8	32.5 - 407.6	1.40 - 18.31
PAAE taken at 540 nm	0.03 - 0.68	0.51, 0.68	9	4	27.0 - 387.8	0.98 - 29.70
Derivatised PAAE taken at 540 nm	0.08 - 0.74	0.63	11	15	20.1 - 177.4	0.86 - 26.63

Table 4: Chromatographic finger print analysis of PAAE at various wavelengths for detection of class of compounds [Lignans]

Name of extract	Parameters					
	Rf range	Rf of Unique loci	No of Peaks	No of Auto Generated tracks	Max Height	Area % range
PAAE taken at 366 nm	0.12 - 0.74	0.39	9	12	11.4 - 101.7	2.41 - 44.58
PAAE derivatised at 366 nm	0.19 - 0.71	0.30	3	8	17.5 - 24.1	8.53 - 51.87
PAAE taken at 540 nm	0.45 - 0.70	0.45	3	6	19.6 - 30.0	19.76 - 50.69

Table 5: Summary of docking analysis of PGE synthase with Phyllanthus compounds.

Compound	Dock score	H-bond	Pi-stacking	Hydrophobic
INDOMETHACIN	-76.97	CYS110A	-	-
Hypophyllanthin	-37.71	-	-	TYR107A, CYS110A, PRO111A

4. CONCLUSION

4.1. Conclusions for effects of formulated PAAE Microemulsion on the carrageenan induced pain- To conclude in the present carrageenan induced acute pain model, we have evaluated the role of PAAE in the reversal and inhibition of the state of acute muscle hyperalgesia.

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Conflicts of interest: We all the authors declare no conflict of interest.

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