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PREFORMULATION OPTIMIZATION OF CATECHIN EXTRACTS FROM ASSAM GREEN TEA AS A CANDIDATE FOR TOPICAL CHEMOPREVENTION

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ABSTRACT

This study reports the solution stability of the main polyphenol component Epigallocatechin Gallate (EGCG) in Assam Green Tea extract to facilitate the further development as topical formulation for cancer chemoprevention. Extraction of green tea was carried out by solvent partitioning method to get maximum amount of water soluble extractives such as EGCG and other catechins. Green tea polyphenols especially EGCG has been reported to be very susceptible to degradation in aqueous medium at room temperature. The stability studies of green tea extract was performed in buffers of different pH, distilled water and methanol to assess the extent of degradation of EGCG with time and the effect of solvent pH on the same. The change in EGCG concentration with time was recorded. An ISO standard simple High Performance Liquid Chromatography (HPLC) method was used to quantify the amount of EGCG in green tea extract. The solution stability of EGCG was found to be prolonged with pH 4.5, which is almost close to pH of the skin. Extract was subjected to freeze drying and kept at 4^{o}C for ease of use in further studies. The extract prepared in pH 4.5 can be successfully used in topical formulation for effective topical chemoprevention.

KEYWORDS: Green Tea Extract, Epigallocatechin Gallate (EGCG), Catechins, Chemoprevention, Skin cancer.

INTRODUCTION

Tea is considered to be a global beverage that possesses inherent antioxidant properties which show understandable health benefits including cancer prevention [Yang et al., 2009]. Green, oolong or black tea are available to consumers depending upon the post-harvest processing

applied to the tea leaves [Sabhapondit et al., 2012]. Various epidemiological and clinical studies have shown a positive relationship between its consumption and disease prevention, for which green tea has received considerable attention in recent past (Katiyar et al., 1992; Cabrera et al., 2006; Yang and Hong, 2013). People consuming green tea is associated with reduced risk of diabetes, cardiovascular disorder (e.g. hypertension, myocardial infarction, atherosclerosis), neurodegenerative diseases (e.g. Parkinsonism and Alzheimer's disease), certain cancers (e.g. gastric, prostrate, breast, cervical) [Dube et al., 2010]. These beneficial effects are largely due to the higher content of catechins in green tea [Arts et al., 2005].

Flavonoids are the major components of total phenolic compounds in tea leaves, which cover almost 90% of dry weight). Among them Flavan-3-ols known as catechins become the predominant one, which cover up to 30% of dry weight. Catechins can be categorized into two isomers: trans-catechins and cis-epicatechins on the basis of the stereo chemical configurations of 3', 4'-dihydroxyphenyl and hydroxyl groups at the 2- and 3-positions of the C-ring. Trans-catechins have two optical isomers: (+)-catechin and (-)-catechin and similarly (-)-epicatechin and (+) - epicatechin are optical isomers of cis-epicatechin. While (-)-catechin can be converted by esterification with Gallic acid to produce the esterified or galloyl catechins: (-)-catechin-3-gallate, (-)- epicatechin-3-gallate, (-)-epigallocatechin-3-gallate and (-) - gallocatechin-3-gallate [Friedman et al.,2005]. Epigallocatechin gallate (EGCG), the main polyphenolic fraction of green tea, acts as an antioxidant, which entraps peroxyl radicals and thus suppresses radical chain auto oxidation. Several recent studies reveal that EGCG is very effective in the treatment of lung cancer, colon cancer and skin cancer [Zaveri, 2006]. The anticancer EGCG inhibit only the growth of cancerous cells, whereas normal cells remain unaffected (Chen et al., 1998; Singh et al., 2011). Formulations with green tea extract will have a stronger effect than any individual tea component due to its synergistic effect with other catechins and caffeine [Wang et al., 2000]. In spite of EGCG being a promising therapeutic agent for many applications, there are currently no data available concerning various aspects of interest that are prerequisite for topical formulation of effective preparations. Therefore, the objective is focused on the preformulation study to determine the stability of EGCG in various solvents that would potentially be suitable for the development of an effective topical formulation for use in preclinical and/ or clinical trials.

MATERIALS AND METHODS

Green Tea (Camellia assamica (Masters)) was collected from the Dibrugarh area of Assam in

India. Epigallocatechin gallate (EGCG) was procured from sigma Aldrich along with polyphenol 60. All others reagents and solvents utilised during the work were of analytical reagent (AR) grade and were used without further purification.

Extraction of Tea Catechins

The extract was obtained by solvent partitioning method. Briefly, known amount of Assam green tea (Camellia assamica (Masters)) was first crushed in mortar, and then hot distilled water was added in to it and kept at 80^{o} C for 20 minutes. Same amount of green tea was extracted twice with same amount of distilled water. Extract was then filtered through Whatman filter paper (Grade 5). The filtrate was partitioned with chloroform (1:1) resulting in removal of caffeine and pigments (Row and Jin 2006). Subsequently, the aqueous layer was extracted twice with equal volume of ethyl acetate and concentrated in a rotary evaporator (Senol and Aydin, 2006; Goodarznia and Govar, 2009; Banerjee and Chaterjee, 2015). The concentrated extract was again hydrated and freeze dried using a freeze dryer (Model: SS1-LYO, Southern Scientific Instruments, Chennai India) to obtain the final extract, which was stored at 4^{o} C in amber coloured vial until required for further experiments. The extract was then subjected to qualitative tests for the confirmation of the presence of tannins and flavanols.

Spectrometric and calorimetric characterization

The obtained extract was then subjected to spectrophotometric analysis considering EGCG as the major component of the extract for further preformulation studies of the extract with the intention of topical administration for cancer chemoprevention. To obtain the UV-VIS spectrum, the extract was dissolved in variety of solvents and the absorption spectra was recorded over a range of 200-800 nm. The λ_{max} value obtained was then correlated with the reported value of reference standard drug (Epigallocatechin gallate) in the same solvent. As epigallocatechin gallate was found in larger quantities (around 40-70%) in green tea extract so λ_{max} value of EGCG (274 nm) was considered for later utilization.

The FT-IR spectra of the pure drug sample was recorded by ATR (Attenuated total reflectance) technique in a FT-IR spectrometer (ALPHA, Bruker, Germany) over a range of 4000 cm^{-1} to 400 cm^{-1}. The drug sample was kept over the lens of the ATR probe and the spectrum was obtained.

The thermal analysis of freeze dried extract was examined by differential Scanning

Calorimetric (DSC) analysis using Jade DSC (Perkin Elmer, USA). The system was calibrated with a high purity sample of Indium. The drug sample (10 mg) was scanned at 10^{o} C/min over a temperature range of 35^{o}C to 250^{o}C in nitrogen gas environment having flow rate of 20 ml/min.

Quantitative characterization

EGCG content present in the extract was quantitatively estimated using waters high-performance liquid chromatography (HPLC) system (Waters 2487, USA) with Nova-Pak 5 µ octadecylsilane column and UV detector set at 278 nm according to specified method of International Standard Organisation. During HPLC analysis, 10 µg/mL solution of the extract was prepared and 10 µL of the solution was injected into the column. The column was eluted with 100% mobile phase A (2% Acetic acid with 9% Acetonitrile in Millipore (type 1) water) for 10 min and then with a linear gradient of 68% mobile phase A and 32% mobile phase B (80% Acetonitrile) for 15 min and hold at this composition for another 10 min with 1mL/min flow rate. The chromatograms were analysed and estimation was done by external standard method by plotting standard curve of catechin standards procured from Sigma Aldrich, USA. The concentration of the EGCG in prepared green tea extract was determined by area of the chromatogram and regression equation of the standard curve. The solvents used for extraction and analysis were of HPLC grade [ISO 14502-2, 2005].

Stability studies

Green tea polyphenols especially EGCG have been reported to be very susceptible to degradation in its free form in aqueous medium at RT. The stability studies of green tea extract was performed in buffer solution of different pH, distilled water and methanol to assess the extent of degradation of EGCG with time and the effect of solvent pH on the same [Batchelder et al., 2004]. Briefly, stock solutions of 100 µg/ml of the green tea extract were prepared in distilled water, methanol, hydrochloric acid buffer of pH 1.2, acetate buffer of pH 4.5 and 5.5 and phosphate buffer of pH 6.8 and pH 7.4. The solutions were then subjected to UV-VIS spectroscopy to assess the absorbance values as well as absorption maxima of EGCG in different solvents and the concentration of EGCG was quantified using standard curve to obtain the initial concentration. Solutions were then kept under constant shaking at 37^{o}C using temperature controlled shaking water bath and the solutions were then again quantified at time intervals of 3, 7, 24, 30 and 48 hours. The change in EGCG concentrations with time was recorded.

Saturation solubility studies

An excess amount of Green tea extract was added to different solvent systems like distilled water, methanol, hydrochloric acid buffer of pH 1.2, acetate buffer of pH 4.5 and pH 5.5 and phosphate buffer of pH 6.8 and pH 7.4. The samples were the shaken continuously in a mechanical shaker for 24 hours at 37± 0.5^{o} C until equilibrium was achieved. After 24 hours the solutions were filtered through Whatman filter paper (Grade 42). The filtrate was diluted 100 times with respective solvent system and the amount was quantified at respective λ_{max} spectrophotometrically for different solvent systems from the respective calibration curves.

Partition coefficient determination

Partition coefficient of Green Tea Extract was determined between equal volumes of three different solvent systems as aqueous phase i.e. distilled water, acetate buffer of pH 4.5, Phosphate buffer of pH 6.8 and n-octanol as the organic phase. These solvent systems would help us to predict the skin permeability of Green Tea Extract form topical gel formulation at normal skin condition. At first, equal volume of two solvents were mutually saturated by shaking at room temperature for 24 hours on a mechanical shaker and then let them stand enough to allow the phases to separate and to achieve a saturation state. After the achievement of equilibrium between the two phases, they were separated and 10 mg of Extract was added to the aqueous phases. Aqueous drug solutions and organic phase were mixed again and shaken gently for 4 hours and then kept for 48 hours to attain the equilibrium. 1ml of the aqueous solution was then recovered and amount of extract was quantified spectrophotometrically after proper dilution with the respective aqueous phase [Sangster, 1997]. Partition coefficient was then determined using the formula: $P = (C_a - C_b)/ C_b$, Where, P = Partition coefficient, C_a= Initial concentration of extract in aqueous phase (µg/ml) and C_b= Final concentration of extract in aqueous phase after equilibrium (µg/ml).

Conflicts of interests none

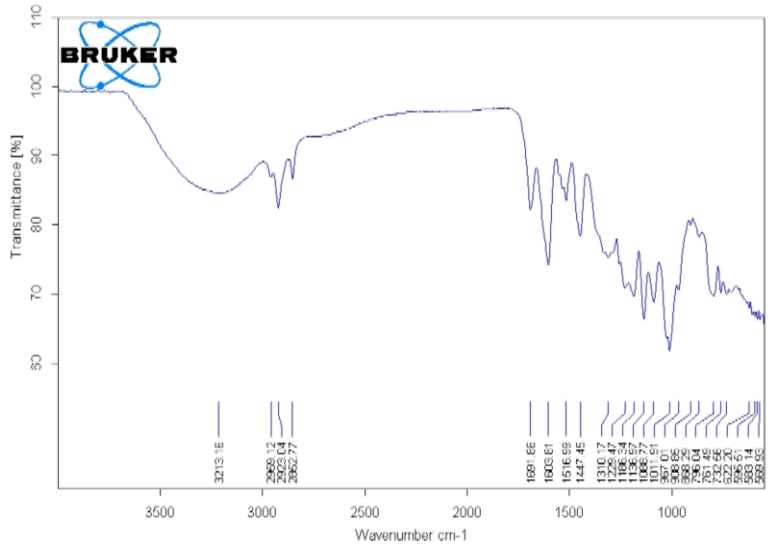

Fig. 1: FT-IR Spectra of Assam Green Tea Extract.

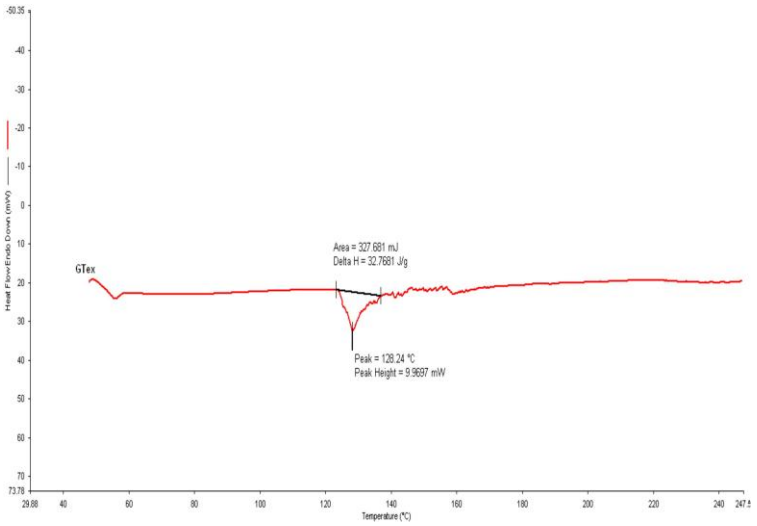

Fig. 2: DSC Thermogram of Assam Green Tea Extract.

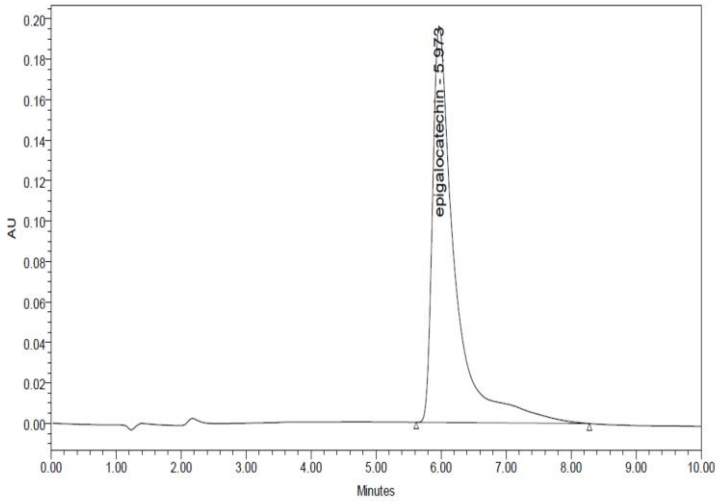

Fig. 3: HPLC Chromatogram of standard Epigallocatechin gallate (EGCG).

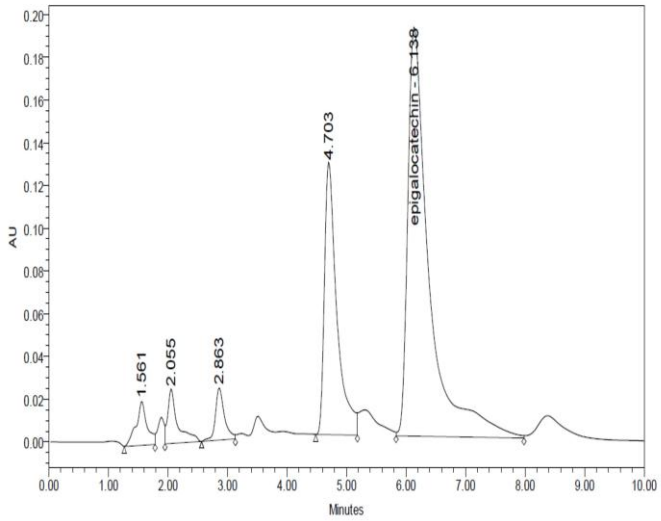

Fig.4: HPLC Chromatogram of Assam Green Tea (Camellia assamica) Extract.

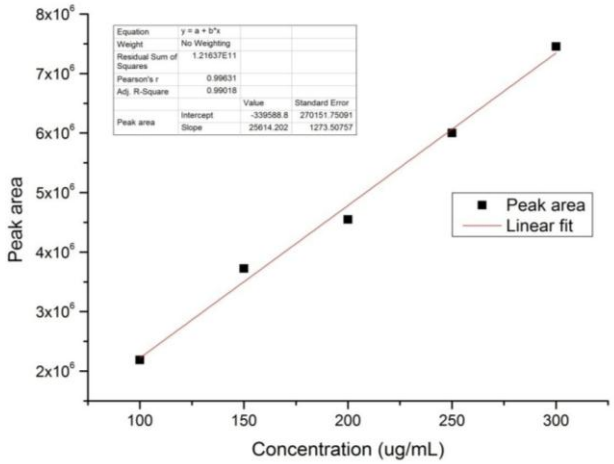

Fig.5: Calibration curve of Epigallocatechin gallate (EGCG).

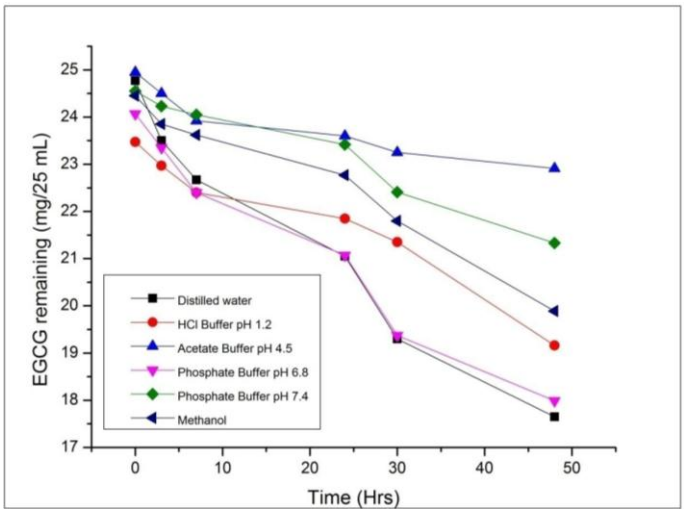

Fig. 6: Comparative stability studies of EGCG in Assam Green Tea Extract in different solvents.

Table 1: Solubility of EGCG in Assam Green Tea Extract in various solvent systems.

Solvent system(s)	Solubility (mg/ml) (mean± SD)
Water	38.71±0.61
Methanol	22.74±0.12
HCl Buffer Solution of pH 1.2	9.54±0.06
Acetate Buffer Solution of pH 4.5	12.85±0.03
Acetate Buffer Solution of pH 5.5	16.71±0.31
Phosphate Buffer Solution of pH 6.8	17.91±22
Phosphate Buffer Solution of pH 7.4	28.46±.16

Table 2: Partition coefficient of Assam Green Tea Extract in different solvent systems.

Solvent system(s)	Partition coefficient (mean±SD)
n-Octanol/water	0.087±0.011
n-Octanol/Hydrochloric acid buffer of pH 1.2	0.20±0.015
n-Octanol/Acetate buffer of pH 4.5	0.286±0.002
n-Octanol/Phosphate buffer solution of pH 6.8	0.12±0.005

RESULTS AND DISCUSSIONS

Extraction

The gravimetric yield of green tea extract was found to be 3.36% after the extract was freeze dried until it reached a constant weight. The extract yield was determined based on the weight of sorted green tea leaves as initial weight and in that respect the yield was found to be at par with the literature values.

The extract was then subjected to qualitative pharmacognostic tests, which confirmed the presence of tannins and flavonols as the major constituents of the extract. The presence of these compounds asserted the presence of desirable green tea polyphenols as well as gallic acid derivatives which have found activity as antioxidants and anticancer compounds. Moreover, the exact values of these polyphenols are required for the preformulation process prior to using the extract to prepare pharmaceutical formulations which demanded a robust and reproducible HPLC method to quantify the polyphenolic contents of the Assam green tea.

The green tea extract was subjected to FT-IR spectroscopy to assess the similarity in the spectra with that of EGCG as a primary compound. From the IR spectra as shown in Fig. 1, it was found that the extract had shown a similar spectra as that of pure EGCG but there was presence of other peaks in the spectra owing to the presence of certain trace compounds but overall the spectra resembles to that of EGCG with characteristic peaks in the region of 3200 - 3600 cm^{-1} but along with that two alien peaks in the region of 2852 cm^{-1} and 2959 cm^{-1} are also there, which are not characteristic of the EGCG spectra. Also there is a slight deviation of the stretching bands in the region of 1100 – 1700 cm^{-1} due to the presence of interfering groups.

The extract was also subjected to thermal analysis by means of differential scanning calorimetry, which provide an insight into the effect of relative compounds on the thermal behaviour of EGCG in a green tea extract and whether it affects the overall thermal stability of EGCG as an individual stakeholder. DSC Thermogram have shown in Fig. 2. The endothermic peak was found to be 128.24^{o} C which is possibly due to the loss of solvent from the solvated form of EGCG and is showing some deviation from the desolvation endotherm of the solvated crystalline EGCG which in fact corresponds to 105.43^{o} C. This might be due to the amorphous phase of EGCG as a component of green tea extract. Although the second endotherm is usually observed at 250^{o} - 260^{o} C which is infact considered the melting endotherm. But the actual characterization is in terms of heat of desolvation in EGCG [Smith et al., 2013].

Quantitative characterization

In this regard, we have commissioned a slightly modified HPLC method for the estimation of the important compounds. However the main focus was on EGCG as it is considered the most potent and most abundant active compound extracted from green tea leaves from Assam.

Initially a calibration curve of pure EGCG was made utilising the same method to validate the linearity and robustness of data. To this effect, 5 different concentrations of pure EGCG were prepared in methanol and injected on to the system and the response in terms of peak area was recorded and the data was plotted between concentration and peak area. The plot showed a linear relationship between the two with a regression coefficient of 0.9901 as depicted in Fig. 5.

Consecutively after the standard curve was prepared, a methanol solution of the green tea extract was injected onto the system using the same method set as that for pure EGCG and the data was collected in terms of retention time and peak area so as to correlate with the time and area of pure EGCG. The percentage of EGCG was found to be 44.05% in the green tea extract. HPLC chromatograms of standard EGCG and prepared green tea extract have been shown in Fig. 3 and Fig. 4, respectively.

Stability studies

The aqueous stability of Assam Green Tea Extract (GTE) was performed to estimate its stability in aqueous solutions with pH ranging from 1.2 to 7.4 in an attempt to find out an optimum condition for the storage of EGCG in solution form for longer durations of time. The Fig. 6 represents the comparative stability of EGCG in different solvent systems. It is clear that the stability of Assam GTE is pH-dependant under the present experimental conditions. The GTE is stable at pH 4.5 and pH 7.4, but unstable in distilled water and pH 6.8. Green tea is an excellent source of polyphenols, known as green tea catechins, a mixture of epicatechin isomers including (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) and EGCG is the major isomer followed by ECG, EGC and EC (Chen and Chan, 1996). Previous studies with Longjing Green Tea Catechins mixture (EGCG content 68% w/w) showed that the catechins were unstable at neutral or slightly alkaline pH and they were stable at the acidic pH. Further studies with individual epicatechin isomers showed the EGCG and EGC were unstable at slightly alkaline pH 7.4, whereas EC and ECG were stable at acidic and alkaline pH (Zhu et al., 1997). The higher content and selective degradation of EGCG might be the reason for less stable nature of Longjing Green Tea Catechins at neutral or slightly alkaline conditions. In contrast, the stability of Assam GTE (experimental EGCG content 44.05% w/w) at acidic pH 4.5 and slightly alkaline pH 7.4 indicates the higher content of EC or ECG in Assam Green Tea Extract. The results clearly demonstrated that EGCG and EGC were selectively degraded

at pH 6.8 and distilled water. The presence of multiple hydroxyl groups in EGCG/ EGC as compared to EC/ ECG has been shown to be more susceptible to oxidation forming semiquinone free radicles since it itself works as an antioxidant at neutral pH (Zhu et al., 1997; Yoshioka et al., 1991). The stability data shown in Fig. 6 clearly indicates that the optimum pH for utilization is pH 4.5. At this acidic pH, the hydroxyl groups are not available in the unionized form and thus are not available for oxidation. But delivery of EGCG at such low pH will be challenging as it can affect the buffering capacity of the media and also it might lose its inherent antioxidant property. So a formulation can be envisaged where a more potent antioxidant like EDTA can be added in trace amounts to prevent the oxidation of the active EGCG itself. However, very low pH 1.2 also have a deleterious effect on the stability of the active compound because of the fact that a very low pH might have catalysed the acidic hydrolysis of the ester linkages found in EGCG and thus have caused the breakdown of the pharmacophore structure itself. Also methanol solution was found to contribute to instability of the compound owing to its ability to contribute for the oxidation process itself.

Saturation solubility studies

The solubility of green tea extract in various solvent and phosphate buffer of different pH was determined. Solubility study shows that the Assam GTE is freely soluble in water and its solubility increases with increasing pH of Phosphate buffer solution and also it has good solubility in methanol. The solubility pattern of the drug depicts its hydrophilic nature (Table 1).

Partition coefficient

Partition coefficient of Assam GTE was determined in n-octanol/water, n-octanol/hydrochloric acid buffer of pH 1.2, n-octanol/acetate buffer of pH 4.5 and n-octanol/phosphate buffer of pH 6.8 at 37 ± 10^{o} C. The results indicate the hydrophilic nature of Assam GTE. The partition coefficient data of GTE in different solvent systems are shown in Table 2. The results clearly demonstrated that the partition coefficient value of EGCG in octanol/acetate buffer at pH 4.5 appears more than that in octanol/phosphate buffer at pH 6.8. In fact, EGCG is likely to be more prone to formation of semiquinone free radicals at pH 6.8 (Yoshioka et al., 1991) than that at pH 4.5 resulting in less distribution into the octanol phase.

CONCLUSIONS

Water based extraction of green tea was carried out to produce Freeze dried extract containing catechins enriched with EGCG. The suitable extraction condition was found to be heating the extract at 80^{o} C for 20 minutes for two successive times with the same amount of green tea. The gravimetric yield of the extract was found to be 3.36% w/w. Qualitative analysis confirmed the intact presence of tannins and flavanols. Quantitative content of EGCG was found to be 44.05% w/w in the Assam GTE which is in agreement with the reported percentage of EGCG of around 40 - 60%. The results of this preformulation study showed that the stability of EGCG is pH dependent and green tea catechins are almost stable at pH 4.5. Based on the present results, an effective formulation can be prepared for topical application and preclinical and/ or clinical trials may be performed.

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