IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
January 2021 Vol.:20, Issue:2
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Isolation of Caffeine from Tea and Preparation of Its Microemulsion to Evaluate Its Various Bioactivities

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Submitted: 05 December 2020
Revised: 26 December 2020
Accepted: 16 January 2021

www.ijppr.humanjournals.com

Keywords: Caffeine, microemulsion, antioxidant, free radicals.

ABSTRACT

Caffeine as a neuromodulator has fascinated attention of researchers across the world. Its various medicinal values & bio activities have been studied. **Objective:** Keeping free radical scavenging activities of the caffeine, the objectives of the study was to isolate caffeine from the tea, validate its various antioxidant activities and prepare microemulsion using the isolated caffeine crystals. **Methods:** Caffeine was extracted by liquid- liquid extraction method using dichloromethane as one of the solvent. Caffeine crystals were further used for studying *in vitro* anti-oxidant studies along with for the preparation of microemulsion. Caffeine microemulsion was prepared by using rice bran oil, tween 80 as surfactant and ethanol as co-surfactant. **Results:** Thin layer chromatography (TLC) with Rf value 0.55 and UV visible spectra with λmax at 272nm confirmed isolation of caffeine crystals. DPPH, the stable free radical was scavenged in a concentration dependent manner, scavenging was directly proportional to caffeine concentration, at 500µg/ml concentration it showed 53% scavenging. Caffeine at a conc. of 500µg/ml inhibited 42.19% lipid peroxidation. Total antioxidant activity, reducing power activity and nitric oxide scavenging potential was also well observed. Oil in water microemulsion was prepared by water titration method. Stability of microemulsion was confirmed by dye solubility test and dilution test. Qualitative DPPH assay with caffeine microemulsion confirmed its free radical scavenging activity. **Conclusion:** In the present study, isolated caffeine crystals showed good antioxidant & free radical scavenging activity and formulated microemulsion was stable with good free radical scavenging activity.

INTRODUCTION

Caffeine a widely researched neuromodulator has adenosine like structure. It is regarded as powerful brain stimulator. Scientists extracted it from seeds and leaves of various plants like guarana berries, guayusa, kola nuts, tea plant and coffee beans yerba mate and cocoa beans. The researcher could also synthesize in labs. Caffeine is a major ingredient in several popular energy drinks. In addition to energy drinks & beverages, many juices, cookies, syrups, waffles, lip balms, cookies, creams, body lotions, shampoos, etc. contain caffeine as one of the primary ingredients. The health claims of caffeine products include energy enhancer, mental alertness, anti-oxidant and anti-aging effect [1].

Novel delivery systems as well as nanoparticle, nano-vesicular systems and microemulsion (ME) improve delivery of the active ingredients [2]. Caffeine Microemulsion (CME) may be a thermodynamically stable, single optically isotropous system of water, oil, and amphiphile. Caffeine has hydrophilicity that restricts its penetration through the skin as well as retention in the skin. Microemulsion formulation will be of huge utility as it will enhance caffeine permeability & retention in the skin. Antioxidant potential of caffeine can protect cell membrane from oxidative damages caused by reactive oxygen species [3]. Antioxidants are of huge benefit to health and these reduce the risk of chronic diseases by ROS including skin cancer, metabolic disorders and cardiovascular diseases [4]. The aim of the research was to isolate caffeine crystals, evaluate their various *in-vitro* antioxidant potential and formulate its stable Microemulsion.

MATERIALS AND METHODS

Isolation and crystallization of caffeine from tea

About 10% of the tea granules and calcium carbonate were added to 500 ml DH_2O water. The mixture was heated at 90ºC for about half an hour. The extract was kept at room temperature and cooled down. The aqueous extract of tea granules was filtered and filtrate was kept at room temperature for some time till it has cooled down and then it was poured into a separating funnel. Then 15ml of dichloromethane was added to the filtrate obtained and transferred it to separating funnel. The contents were rigorously mixed and then were allowed to settle down for 60 min until a mixture separated into two separate layers. The lower layer of dichloromethane was collected in a conical flask and the aqueous layer was again subjected for the extraction with dichloromethane. Anhydrous magnesium sulphate was then

added to dichloromethane fraction and was kept undisturbed for 10 minutes. The dichloromethane fraction was kept overnight at room temperature for evaporation; after which, light green coloured crude caffeine powder was obtained [5].

Crystallization of caffeine

Crude caffeine extract was dissolved in the minimum quantity of warm methanol (5ml). If the crude caffeine does not dissolve in it, then an additional 5 mL methanol was added. On dissolution, the content of the flask was allowed to reach room temperature and then place it at 4°C for crystallization. It may become essential to scratch the sides of the flask in order to induce effective crystallization. Crystals so obtained were air-dried.

Spectrophotometric analysis

In the different samples of tea, the caffeine alkaloid content was determined with UV-VIS Spectrophotometer. Distilled water was used to dilute the samples as well as blank. The absorption maxima, λmax, of the test sample, was recorded. A standard of caffeine was made by the dissolution of 10 mg caffeine in 100 ml distilled water [6].

Thin layer chromatographic analysis of caffeine

Preparation of silica plates for TLC

The silica slurry for TLC was prepared by mixing 20g of Silica gel, 4 g of Calcium Sulphate as a binder in 50 ml of Distilled water. The slurry was spread on a glass plate and was permitted to dry at room temperature. The dried silica plate was activated in the oven at 110°C for 60 min [7].

Sampling of caffeine on TLC plates

Caffeine samples (10µL) were spotted on 1 cm from the bottom of the TLC plate. The plates were placed inside a saturated thin layer chromatography chamber, containing mobile phase mixture consisting of ethyl acetate:methanol:acetic acid: 80:10:10. The mobile phase was allowed to run till the top end of TLC plate. The TLC plates were kept in saturated iodine chamber to mark & identify position of caffeine spots.

DPPH scavenging assay

Different caffeine samples ranging from 10µg - 200µg were reacted with 0.5 mM DPPH solution prepared in methanol. The absorbance of all the test tubes was taken at 517nm using a UV visible spectrometer [8]. The percent DPPH scavenging activity was obtained by applying the formula as given below:

DPPH scavenging activity (%):

$$\frac{(\text{Absorbance of Control} - \text{Absorbance of Sample}) \times 100}{\text{Absorbance of Control}}$$

Lipid peroxidation assay

Caffeine stock solution (2mg/ml) was prepared in methanol and serially poured into the test tube in the concentration range of 10µg - 200µg. Then, distilled water added to all test tubes to make 1 ml volume of each test tube. After the distilled water was added, egg yolk (10%) was added to each test tube and then 0.1 ml of ferrous sulphate (15mM) was added to them. The reaction mixture was incubated at 37° C for 1 hour. After incubation, precipitation was done by adding 1.5 ml of chilled TCA reagent (10%) in all test tubes. Then, reaction mixture is centrifuged at high speed for 20 minutes. 1.5 ml of supernatant was reacted & incubated with 1.5 ml of TBA reagent at 100° C in the water bath for 30 minutes. After incubation, absorbance was taken at 535 nm. The antioxidant activity was recorded by a plot was made between percent lipid peroxidation activity and sample concentration (µg) [9]. The percent lipid peroxidation activity was deduced by applying the following formula:

Lipid peroxidation activity (%):

$$\frac{(\text{Absorbance of Control} - \text{Absorbance of Sample}) \times 100}{\text{Absorbance of Control}}$$

Assay of total antioxidant activity:

Total antioxidant activity of caffeine was measured by the method of reduction of phospho-molybdate. The reaction mixture containing ammonium molybdate and sodium phosphate was with reacted with caffeine at acidic pH at 90^0 C. The optical density was evaluated at 695nm [10].

Nitric oxide scavenging activity

Nitric oxide scavenging activity was evaluated with the caffeine stock solution of 2mg/ml and the caffeine concentration range was between 10µg - 200µg. Then, the volume was made with normal saline (0.9%) to 1 ml in each test tube and sodium nitroprusside (2ml, 100mM) was added. All the reaction tubes were then incubated for 2hrs at room temperature. After incubation, 1.5 ml of incubated solution was taken and reacted with 1.5 ml of griess reagent (0.1% naphthylene + 2% H_3PO_4 + 1% sulphanilamide). Then, the test tubes containing reaction mixture were incubated again at RT for half an hour. After incubation, absorbance was measured at 546 nm [11]. A graph was plotted with percent of nitric oxide scavenging activity versus caffeine concentration (µg). The percent nitric oxide scavenging activity was determined using the following calculation:

Nitric oxide scavenging activity (%):

$$\frac{(\text{Absorbance of Control} - \text{Absorbance of Sample}) \times 100}{\text{Absorbance of Control}}$$

Reducing power assay

Previously prepared caffeine solution was utilized for reducing power assay. The caffeine concentration range used for the assay was 10µg - 200µg. the reaction mixture containing 1% potassium ferricyanide were incubated at 50° C for 20 minutes, then added 2.5 ml of chilled TCA (10%) was added in and centrifuged at high speed for 10 minutes. After centrifugation, supernatant was taken from each test tube and transferred to fresh test tubes individually. Then to each test tube, ferric chloride (0.1%) was added [12]. The reaction mixture containing tubes were left for 5-10 minutes, absorbance was measured at 700 nm.

Preparation & characterization of microemulsion

Microemulsion was prepared by using rice bran oil as sample, tween 80 as surfactant, ethanol as co-surfactant and distilled water during titration. Surfactant: co-surfactant (Smix) ratio was set to 4:1 and added rice bran oil to it in 9:1 ratio (Smix: oil). The final volume was adjusted to 80 ml. Then, the whole mixture was stirred over laboratory stirrer at very high speed along with the dropwise addition of distilled water containing caffeine. The titration was continued until turbid solution was made. Then, the distilled water was again added drop wise till a

clear solution was made known as microemulsion [13]. The final microemulsion had 1% caffeine.

Characterization of Caffeine Microemulsion

The characterization of caffeine microemulsion was done with the help of *in-vitro* tests.

Dilution test – In this test, distilled water was supplemented to the microemulsion with continuous stirring at high speed. 10-20 ml water was added to the microemulsion and observed the appearance of microemulsion [14].

Dye solubility test – In this test, one drop of ink was added to 10 ml of microemulsion and mixed well. Then, the appearance of microemulsion was observed for the presence of either clumps or clear solution [14].

RESULTS

The objective of our study was to isolate caffeine from the Tea and check its antioxidant activities using various *in-vitro* assay methods and prepare a stable microemulsion. The results obtained are given below.

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Phytochemical studies

Thin layer Chromatography of methanolic tea extract was performed using Ethyl acetate: methanol: acetic acid (80:10:1) solvent system. The chromatogram showed the presence of caffeine in the tea samples we procured from the market. The Rf value for caffeine spots was 0.55.

Extraction of caffeine

As a thin layer chromatography of the tea extracts showed the presence of caffeine, the samples were further processed extraction of caffeine. Tea samples were extracted with boiling water for 30 min as mentioned in detail the material and method section. The aqueous tea extract thus obtained was allowed to cool down and filtered through the muslin cloth to remove unwanted tea granules. Then filtered aq. extract was further filtered through the Whatman filter paper to get a clear extract free from any residual tea particles. The filtered aqueous extract was poured into a separating funnel and then to isolated caffeine, dichloromethane was added to the separating funnel filled with aqueous tea extract. The separating funnel was shook rigorously for 15 min and allowed to settle for some time. The

separating funnel had two layer, one layer of aq. Tea extract and 2nd layer of dichloromethane with caffeine dissolved into it. Once the two layers were clearly separated, DCM layer was collected in a beaker. The aqueous tea extract in the separating funnel was again extracted DCM and entire process was repeated. After rigorous mixing, separating funnel was kept still on its stand and two solvents were allowed to settle. After some time, DCM layer was again collected in a beaker. Both the samples of DCM layer were mixed and TLC was performed to confirm extraction of caffeine from the aqueous Tea extract (Fig). Caffeine spots were clearly observed on thin layer chromatography plates (Fig).

Figure No. 1: Extraction of Caffeine

Isolation of caffeine crystal

Caffeine has high solubility in dichloromethane. Once we performed liquid extraction, alkaloid from the liquid tea extract got solubilized in methylene chloride. The DCM fraction was allowed to evaporate at temperature for nightlong. A greenish crude extract of caffeine was obtained. The dried extract was dissolved in methanol (10ml) and allowed to chill down. The methanol crude caffeine extract was kept at 4°C for slow evaporation of methanol and to initiate crystallization of caffeine. Greenish coloured crystals of crude caffeine were formed. These crystals were once more dissolved in methanol and

kept at 4ºC for re-crystllization. The recrystallization step was repeated till we get white needle formed, shining pure caffeine crystals.

Spectroscopic analysis of caffeine

The UV spectra of various caffeine samples and fractions, such as tea, dichloromethane fraction, and caffeine crystals were evaluated within the spectral range of 200 - 500 nm using UV spectrometer. The spectra were recorded at a spectral bandwidth of 1 nm. The absorption max (Lambda max) for caffeine was 272nm.

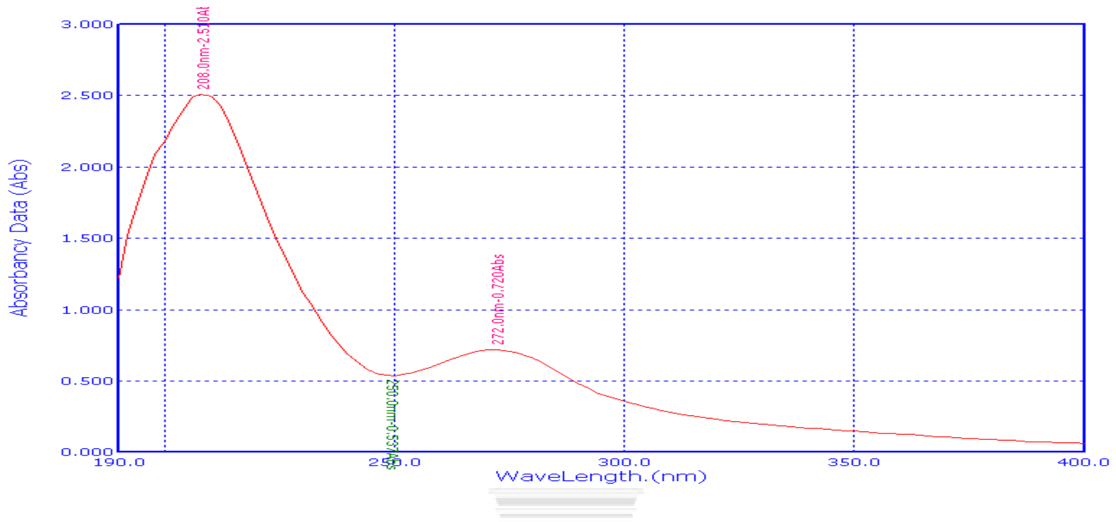

Figure No. 2: UV visible spectra of aqueous extract of tea granules

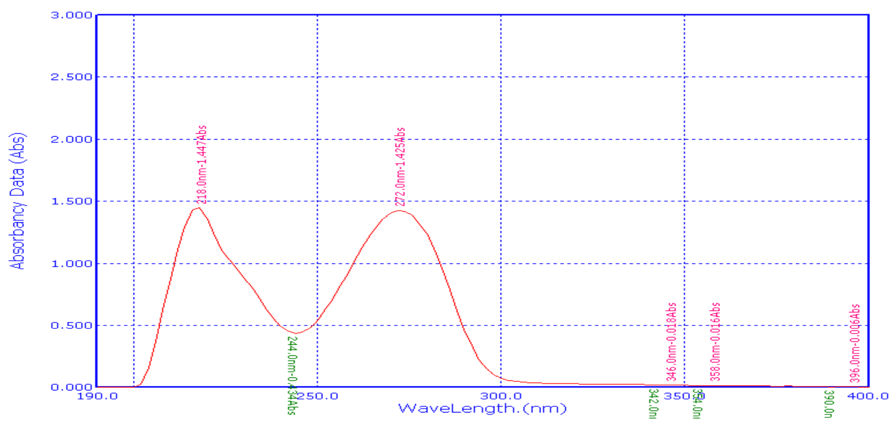

Figure No. 3: UV Visible spectra of Dichloromethane fraction

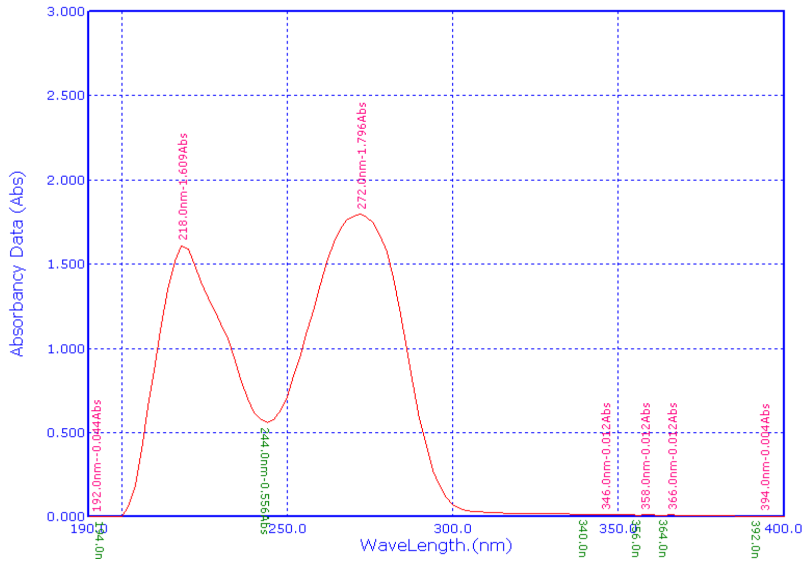

Figure No. 4: UV Visible spectra of caffeine crystals

DPPH scavenging assay

The Alkaloid, caffeine caused a significant reduction of oxidized DPPD to reduced DPPH because of which purple-colored DPPH became pale yellow. Caffeine was found to be effective in scavenging DPPH stable radicals. The free radical scavenging and antioxidant potential caffeine increased with a rise in caffeine concentration as indicated by concentration dependent change in absorbance. At a caffeine conc. of 10 µg/ml, 32.23% scavenging of DDPH radical was observed and at a conc. of 200 µg/ml, approximately 50% scavenging of DDPH radical was observed.

Figure No. 5: Reduction of DDPH by caffeine

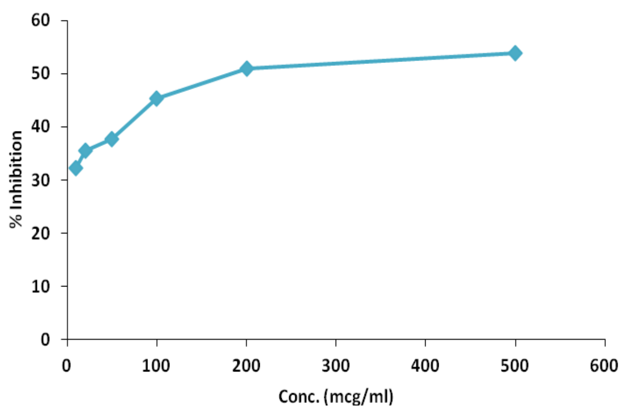

Figure No. 6: Percentage scavenging of DPPH

***In vitro* lipid peroxidation**

ROS initiate lipid peroxidation, so the formation of Thiobarbituric acid reactive substances (TBARS) is computed to evaluate extent of lipid peroxidation. Unsaturated lipids that are present in the egg yolk undergo peroxidation, in the presence of reactive oxygen species. Unsaturated lipids of egg yolk were reacted; ascorbate/$FeSO_4$ to cause peroxidation and absorbance of the reaction mixture was measured at 532nm.

Caffeine crystal exposure resulted a significant inhibition in lipid peroxidation, and the inhibition increased with an increase in caffeine concentration. At 20 µg/ml concentration of caffeine, 10.98% inhibition of TBARS formation was detected, and at a concentration of 500(µg/ml) an inhibition of 42.19% TBARS formation was observed.

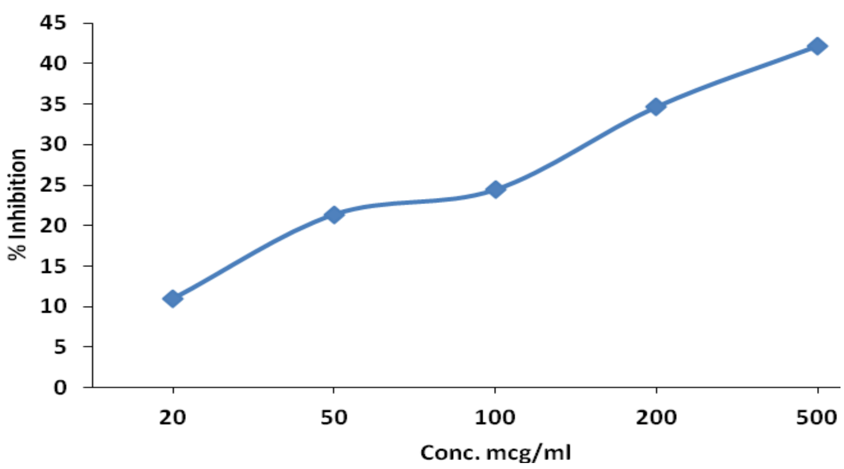

Figure No. 7: *In-vitro* Lipid peroxidation assay

Total antioxidant activity

The overall anti-oxidant activity of caffeine crystals was estimated by the method of ammonium molybdate, and an increase in optical density indicated an increase in total antioxidant activity. Caffeine showed very good total anti-oxidant potential as shown by an increase in absorbance (OD). At 20 µg/ml caffeine concentration, the mean OD was 0.0395, and the absorbance of the mixture increased with a rise in concentration. At 500 µg/ml, caffeine concentration absorbance observed was 0.542.

Effect on total antioxidant activity

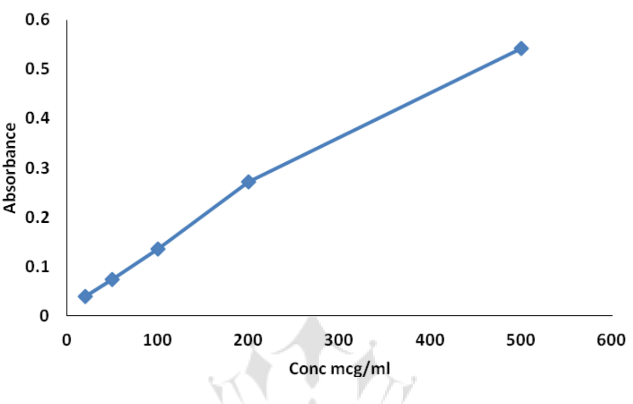

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Figure No. 8: Total antioxidant activity

Nitric oxide radical scavenging

Nitrite radical scavenging assay was evaluated with extracted caffeine crystals at a varied concentration range (10 - 500 μg/mL). The reduction in absorbance was observed and plotted as Nitric oxide radical scavenging (absorbance) versus the concentration of the caffeine crystals, as illustrated in Figure 9. Nitrite radical was formed from sodium nitroprusside at physiological pH in an aqueous solution. Caffeine crystals could scavenge the generation of Nitrite radicals. The antioxidant activity augmented with an increase in the concentration of caffeine. The maximum nitric oxide free radical scavenging and potency was observed at 500 µg/ml.

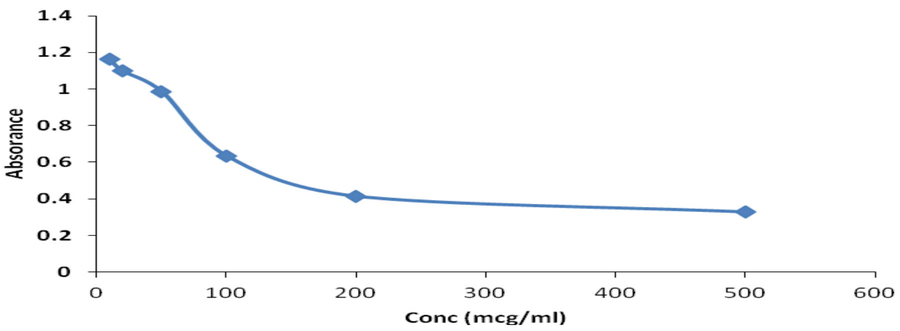

Figure No. 9: Nitric oxide radical scavenging activity

Reducing power assay

The ability to reduce any compound is a critical indicator potent antioxidant potential. The reduction of ferricyanide complex into the ferrous form proves anti-oxidant characteristics of the alkaloid crystals. Thus, the formation of Fe^{2+} was analyzed by the production of Perl's Prussian blue at 700 nm due to the reduction reaction caused by caffeine. The proportional increase in OD was observed with rise in the concentration of caffeine, indicating that the caffeine crystals could reduce potassium ferricyanide (Fe^{3+}) to produce potassium ferrocyanide (Fe^{2+}) that in turn produce ferric-ferrous conjugate on reaction with ferric chloride. As the concentration of caffeine is increased, there was more formation of the ferric-Ferrous complex as indicated by increase in absorbance at 700nm.

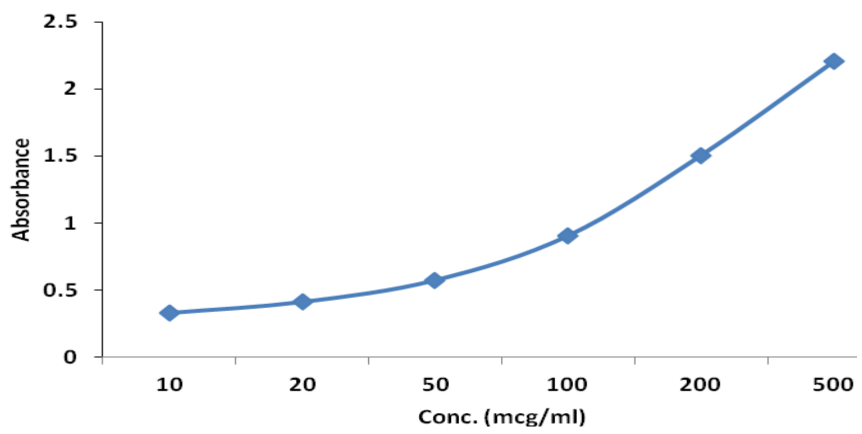

Figure No. 10: Reducing Power assay

Caffeine microemulsion preparation

Microemulsion of caffeine was also prepared using caffeine crystals extracted from tea granules. The caffeine microemulsion during initial formation turbid in appearance and cream

in color but as the titration with DH_2O was continued till colour of microemulsion changes to light-yellowish, transparent & viscous form as shown in Figure 11.

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Figure No. 11: Caffeine containing microemulsion

Dye solubility test – Dye solubility test of caffeine microemulsion was performed to review type and stability of microemulsion. During this test, the dye was completely dispersed & solubilized in the microemulsion. It showed that it was oil in water microemulsion as dye was homogenously immersed in it.

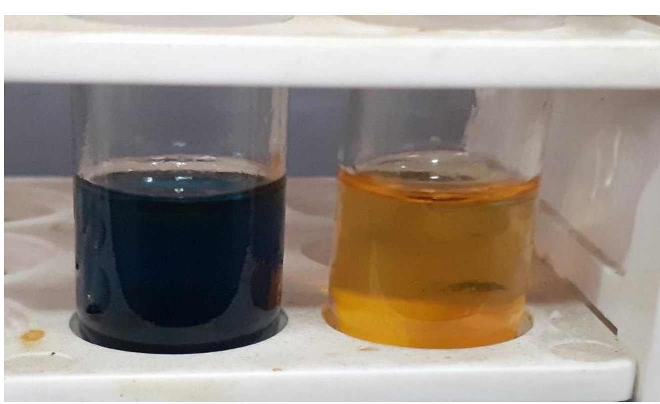

Figure No. 12: Caffeine Microemulsion: Dye Solubility test

Dilution test – Dilution test was conducted with the continuous phase of caffeine microemulsion i.e. distilled water (DH2O) was added to microemulsion and any change in its form was observed. No precipitation or phase separation in microemulsion was observed on dilution with distilled water indicating that it was a stable microemulsion.

Qualitative DPPH assay of caffeine microemulsion

Qualitative DPPH assay was performed on caffeine microemulsion. To the DDPH (1mM prepared in methanol) 50µl and 100 µl of caffeine microemulsion was mixed and change in DPPH color was recorded. The original purple of the DPPH solution was completely changed to pale yellow color as shown in the figure attached below clearing suggesting effective free radical scavenging potential of our microemulsion formulation.

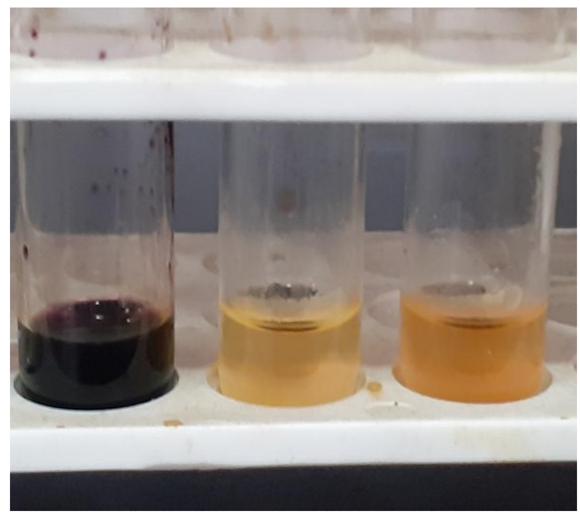

Figure No. 13: Caffeine Microemulsion: Qualitative DPPH assay

DISCUSSION

Caffeine extracted from the tea granules was evaluated using TLC and UV visible spectroscopy. The aqueous extract, DCM fraction and caffeine crystals were used for spectrometric analysis done between the wavelength range of 190 nm to 400nm. The λmax was recorded at 272nm and these results correlated well with those reported in the literature. Belay *et al* determined caffeine content in Arabic coffee beans and aqueous solution of caffeine crystal had absorption maxima at 272nm [6]. Bhawani *et al* determined caffeine content in various pharmaceutical formulations using UV spectrometer. They measured spectra in the range of 225 to 285 nm with intervals of 5nm. They also reported λmax for water extract of caffeine at 272nm [15]. They concluded that spectrophotometric analysis is

easy, validated and accurate method for the determination of caffeine in various samples as well as in mixture of samples. Vichare *et al.* determined concentration of caffeine in various formulations using UV spectrophotometry and reported λmax at 273 nm [16].

Crystallization of caffeine

Scientists developed and used various methods/modified methods for extraction of caffeine and development of needle shaped crystals. Brun *et al* developed caffeine crystals by supercritical antisolvent assay method and the method enhanced product purity considerably. Researchers also reported that temperature and duration of extraction process are critical points to enhance yield and purity of the crystals [17]. Pradeep *et al* extracted caffeine from tea leaves and black coffee beans using liquid liquid extraction. They extracted caffeine with 90% purity and the yield was appx. 5 %. The content of yield varies in different varieties of tea and it could be due to difference in climatic conditions and soil conditions [18].

Mohammadi *et al*. used microwave and ultrasound assisted extraction technique for caffeine purification from Iranian green tea leaves. These modified extraction methods had efficiency of 85-95% [19]. In the present research project, aqueous extract of tea was treated with dichloromethane in a separating funnel. The aqueous content was further extracted twice with DCM and was kept at room temperature for overnight. Light Green colored needle shaped crude crystal of caffeine were formed. The crystals of crude caffeine so obtained were dissolved in minimal quantity of methanol and kept at low temp for crystallization. These crystals were recrystallized until we received white colure caffeine crystals. The white caffeine crystals were powered to form a white colored caffeine powder. The UV visible spectral analysis of the caffeine powder was performed and observed λmax of the crystal was 272nm. The results correlated very well with those reported in the literature.

Free radical scavenging & antioxidant studies of caffeine

Caffeine a purine alkaloid is formulated in many food products and supplements. People consume it through coffee, tea, coca and chocolates etc. Researchers reported its antioxidant potential contributing in protection of liver and kidney damages caused by oxidative stress and generation of peroxides. In the current research project, various *in-vitro* antioxidant assays were used to determine antioxidant potential of the caffeine isolated from tea granules. Fresh Tea leaves processing involved number of steps such as steaming and baking then

drying before these are packaged as tea granules [20]. The objective is to check *in-vitro* antioxidant potential caffeine extracted from processed tea granules available in the market.

As mentioned above, caffeine on reaction with DPPH radical, caused discoloration of DPPH dissolved in methanol. The decrease in purple color of DPPH solution confirmed strong free radical scavenging of potential of caffeine crystals. Once the qualitative DPPH assay data was available a dose dependent study was performed. A steady reduction in OD was observed as the amount of caffeine was increased. At a caffeine conc. of 10 µg/ml, 32.23% scavenging of DPPH radical was observed and at a conc. of 200 µg/ml approximately 50% scavenging of DPPH radical was observed. Results correlated well with literature reported data, Nadanasabapathi *et al* also reported DPPH scavenging with caffeine [20].

Unsaturated lipids are susceptible to lipid peroxidation as they react with various ROS. In this study, the unsaturated lipids present in egg yolk were incubated with ROS generation system, ascorbate/$FeSO_4$, and lipid peroxidation inhibitory activities of caffeine were studied by measuring absorbance at 532 nm. At a low conc. of 20 µg/ml, we observed 10.98% inhibition of TBARS formation and at maximum conc. of 500 µg/ml, an inhibition of 42.19% TBARS formation was observed. The study results clearly suggested that caffeine crystals isolated from tea inhibited lipid peroxidation and inhibition increased with increase in conc. of caffeine. Nadanasabapathi *et al* studied effect of caffeine and caffeine + caffeic acid combination on lipid peroxidation using liver homogenate as a resource of unsaturated lipids. They also reported significant inhibition of lipid peroxidation by caffeine crystals and they reported it to be comparable to ascorbic acid [20]. Devasagayam *et al* showed that alkaloid effectively inhibited lipid peroxidation (LPO), at millimolar concentrations [21]. Azam *et al* suggested that caffeine and its metabolites might effectively contribute in chemopreventive & antioxidant properties of caffeine-containing drinks & beverages [22].

The total antioxidant potential was determined by the method of phosphomolybendenum, the principle is, reduction of Phosphate-Mo (VI) to Phosphate Mo (V) by caffeine that at acidic pH leading to the formation of a bluish-green colored phosphate/Mo (V) complex. It is suggestive of reducing potential of alkaloids like caffeine. At 20 µg/ml caffeine concentration, the mean absorbance was 0.0395 and absorbance of the mixture amplified with an increase in concentration. There was a proportional increase in absorbance at each increased concentration. Afify *et al* evaluated antioxidant properties of caffeine containing

products. They reported extent of antioxidant activity was dependent on concentration and duration of the reaction [23].

Nitric oxide (NO) is crucial in accelerating macrophage cytotoxicity, management of blood pressure, and neurotransmission. As NO radicals are produced in the cell, it may react with superoxide anions to generate peroxynitrite, a strong oxidizing and nitrating molecule. The excess generation of NO by inducible nitric oxide synthase (iNOS) has been linked with the progression of numerous diseases such as diabetes, cancer, cardiovascular diseases and renal diseases. Xu *et al* reported nitric oxide radical scavenging activity of aqueous tea extracts of various varieties of tea like pu-erh tea, green tea, black tea and chemical constituents such as catechins, browning and caffeine. They reported dose-dependent scavenging of NO radicals by tea constituent, and most effective was reported to be caffeine [24].

Reducing impact of caffeine may be the contributor for its potent antioxidant activity. The caffeine crystals could reduce potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which in turn reacts with ferric chloride to yield ferric-ferrous complex. Researchers evaluated reducing power capabilities of caffeine and caffeine + caffeic acid combination. They reported more reducing power activity in the presence of caffeic acid than caffeine alone [20].

In-vitro antioxidant parameters evaluated in the current research study clearly establish free radical scavenging potential of caffeine. Many ailments are caused by oxidative stress and damages caused by free radical. A compound like caffeine with effective anti-oxidant properties can be of high therapeutic value and of health benefits.

Caffeine microemulsion

New Drug Delivery systems like microemulsions improve drug delivery through and into the skin. Microemulsions can be referred as system consisting of water, oil and surfactant which is thermodynamically stable and optically; it has isotropic single phase. Microemulsion are of huge benefit to formulation researcher as these are easy to formulate, thermodynamically stable, improve the delivery of the drugs, improves penetration of the drug through the skin and can be used to formulate hydrophilic and hydrophobic active molecules. Due to these factors, Microemulsions have attracted the interest of many formulation scientists.

In the current study, we formulated caffeine micro-emulsion using water, rice bran oil, tween 80 and ethanol. Rice bran was utilized in the formulation as due to its strong anti-oxidants

potentials and micronutrients present in it. Caffeine along with antioxidant constituents of rice bran oil can form a synergistic combination. The prepared micro-emulsion was further analyzed with dye test, dilution test and centrifugation methods. The dye test showed homogenous distribution of the dye without any phase separation or precipitation. Similarly, dilution test as well as centrifugation tests established stability of the micro-emulsion with no precipitation or phase separation after dilution or centrifugation. The oil in water microemulsion containing caffeine formulated in the current study was stable. The UV visible spectroscopy of the microemulsion detected caffeine peaks & confirmed no deterioration of caffeine during formulation. In the Qualitative DPPH assay, change of purple DPPH solution to pale yellow colored solution confirmed antioxidant activity of the prepared caffeine microemulsion.

Gupta *et al* formulated caffeine microemulsion using poly phenon 60 and reported effective antioxidant and antimicrobial potential of the prepared microemulsion [13]. Huixian *et al* reported preparation of 1% caffeine (CAF) containing topical microemulsion (ME). It improved its curative efficacy on UVB-induced carcinogenesis [25]. Bolzinger *et al* formulated alcohol free oil in water caffeine microemulsion [26]. Microemulsions have been widely accepted topical delivery system that improved drug delivery by manipulating both intra- & intercellular pathways.

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CONCLUSION

In conclusion, caffeine crystals isolated from tea granules showed antioxidant & free radical scavenging potential and formed a stable & homogenous microemulsion.

AUTHORS CONTRIBUTION

All authors contributed equally.

CONFLICT OF INTEREST

Authors do not have any conflict of interest.

REFERENCES

1. Cappelletti S, Daria P, Sani G, Aromatario M. Caffeine: Cognitive and Physical Performance Enhancer or Psychoactive Drug? Current Neuropharmacology 2015;13: 71–88. doi: 10.2174/1570159X13666141210215655.

2. Nastiti CMRR, Ponto T, Abd E, Grice JE, Benson HAE, Roberts MS. Topical Nano and Microemulsions for Skin Delivery. Pharmaceutics 2017;9(4):37. doi: 10.3390/pharmaceutics9040037.
3. Metro D, Cernaro V, Santoro D, Papa M, Buemi M, Benvenga S, *et al*. Beneficial effects of oral pure caffeine on oxidative stress. J Clin Transl Endocrinol 2017;10:22-27. doi: 10.1016/j.jcte.2017.10.001. eCollection 2017 Dec.
4. Song F, Qureshi AA, Han J. Increased caffeine intake is associated with reduced risk of basal cell carcinoma of the skin. Cancer Res 2012;72(13):3282-9. doi: 10.1158/0008-5472.CAN-11-3511.
5. Choung MG, Lee MS. Optimal extraction conditions for simultaneous determination of catechins and caffeine in green Tea leaves. Food Science and Biotechnology 2011; 20(2): 327-333.
6. Belay A, Ture K, Asfaw A, Redi M. Measurement of caffeine in coffee beans with UV/vis spectrometer. Food Chemistry 2008; 108 (1): 310-315.
7. Palacios C , Maria L, Salatino F, Salatino A. TLC Procedure for Determination of Approximate Contents of Caffeine in Food and Beverages. World Journal of Chemical Education 2017; 5: 148-152.
8. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. Journal of Agriculture & Food Chemistry 2005; 53(6):1841-1856.
9. Kumar PV, Sashidhara S, Kumar MM, Shidhara BY. Effect of *Luffa echinata* on lipid peroxidation and free radical scavenging activity. Journal of Pharmacy and Pharmacolog 2000; 52(7):891-4.
10. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Analytical Biochemistry 1999; 269(2): 337-341.
11. Balakrishnan N, Panda AB, Raj NR, Shrivastava A, Prathani R. The evaluation of nitric oxide scavenging activity of *Acalypha indica* Linn Root. Asian Journal Research Chemistry 2009; 2(2): 148–150.
12. Oyaizu M. Studies on products of browning reaction: antioxidative activity of products of browning reaction prepared from glucosamine. Japan Journal Nutrition 1986; 44(6): 307–315.
13. Gupta S, Bansal R, Ali J, Gabrani R, Dang S. Development and Characterization of Polyphenon 60 and Caffeine Microemulsion for Enhanced Antibacterial Activity. Biomed Research International 2014; P7. 932017. doi: 10.1155/2014/932017.
14. Roland I, Piel G, Delattre L, Evrard B. Systematic Characterization of Oil-In-Water Emulsions for Formulation Design. International Journal of Pharmaceutics 2003; 263(1): 85-94.
15. Bhawani SA, Fong SS, Ibrahim MNM. Spectrophotometric Analysis of Caffeine. International Journal of Analytical Chemistry 2015; Article ID 170239, 7 pages http://dx.doi.org/10.1155/2015/170239.
16. Vichare V, Mujgond P, Tambe V, Dhole SN. Simultaneous spectrophotometric determination of paracetamol and caffeine in tablet formulation. International Journal of PharmTech Research 2010; 2(4): 2512-2516.
17. Brun GW, Martín A, Cassel E, Vargas RMF, Cocero MJ. Crystallization of Caffeine by Supercritical Antisolvent (SAS) Process: Analysis of Process Parameters and Control of Polymorphism. Crystal Growth & Design 2012; 12(4):1943-1951.
18. Pradeep S, Rameshaiah GN, Hadagali A, Chandraprasad M. Caffeine extraction and characterization. International Journal of Current Research and Review; 2015; 7: 16-19.
19. Mohammadi VG, Zamani B, Afsharpour M, Mohammadi A. Extraction of caffeine and catechins using microwave-assisted and ultrasonic extraction from green tea leaves: an optimization study by the IV-optimal design. Food Science and Biotechnology 2017; 26 (5) : 1281-1290.
20. Nadanasabapathi S, Rufia. J, Manju V. *In vitro* free radical scavenging activity and bioavailability of dietary compounds caffeine, caffeic acid and their combination. International Food Research Journal 2013; 20(6): 3159-3165.
21. Devasagayam TP, Kamat JP, Mohan H, Kesavan PC. Caffeine as an Antioxidant: Inhibition of Lipid Peroxidation Induced by Reactive Oxygen Species. Biochimica *et* Biophysica Acta 1996; 1282 (1): 63-70.
22. Azam S, Hadi N, Khan NU, Hadi SM. Antioxidant and Prooxidant Properties of Caffeine, Theobromine and Xanthine. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research 2003; 9(9): 325-30.

23. Afify AEMR, Shalaby EAA, El-Beltagi HS. Antioxidant activity of aqueous extracts of different caffeine products. Not Bot Horti Agrobo 2011; 39(2) : 5071-5078.
24. Xu Y, Zhao H, Zhang M, Li CJ, Lin XZ, Sheng J *et al*. Variations of Antioxidant Properties and NO Scavenging Abilities during Fermentation of Tea. International Journal of Molecular Sciences 2011; 12(7):4574-4590.
25. Huixian Ma, Meng Yu, Mingzhu Lei, Fengping Tan, Nan Li. A Novel Topical Targeting System of Caffeine Microemulsion for Inhibiting UVB-Induced Skin Tumor: Characterization, Optimization, and Evaluation. AAPS PharmSciTech 2015; 16 (4): 905-913.
26. Bolzinger MA, Briancon S, Pelletier J, Fessi H, Chevalier Y. Percutaneous release of caffeine from microemulsion, emulsion and gel dosage forms. European Journal of Pharmaceutics and Biopharmaceutics 2008; 68(2): 446-451.