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Lutein extraction from marigold petals by surfactant-free microemulsion technique

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Abstract

Marigold (*Tagetes erecta* L.) is rich in bioactive compounds such as lutein, phenolics, and flavonoids. This work aimed to investigate the effect of water concentration in a binary phase mixture of triacetin (TriA) and ethanol on the formation of a surfactant-free microemulsion for lutein extraction, as well as the antioxidant activity of the extract obtained. The water concentration in the binary phase was varied at 2%, 4%, 6%, 8%, and 10%. The results showed that the highest lutein content was obtained with a 2% water concentration. The total phenolic content was highest at water concentrations of 2% and 6%, which may be associated with enhanced antioxidant activities, including DPPH radical scavenging and Fe-chelating activities. By using a fuzzy analytical method to evaluate the performance of each ternary phase, the 2% water concentration mixture achieved the highest overall performance score. Additionally, the FTIR spectrum of this obtained extract showed a characteristic peak at 960 cm^{-1}. These findings pointed out that the surfactant-free microemulsion composed of ethanol, TriA, and water is an alternative green solvent system for lutein extraction from marigold petals.

1. Introduction

Marigold (*Tagetes erecta* L.) is known as American marigold, containing large flowers that have yellow, orange, and golden color. Marigold has been used as a traditional medicine (Nadakarni 1954; Kadam *et al*., 2013). Marigold is rich in bioactive components, including phenolics, terpenoids, tocopherols, quinones, coumarins, and volatile oils. Among the carotenoids, lutein belongs to the xanthophyll family, which is one of the two major carotenoid families and contains oxygen (Kalariya *et al*., 2012). Moreover, it has been reported that it can prevent heart disease, improve mental and cognitive abilities, lessen nerve damage, stop cellular lipid peroxidation, and have anti-inflammatory, anti-angiogenic, anti-mutagenic, and anti-cancer properties (Koushan *et al*., 2013; Eggersdorfer and Wyss, 2018).

Lutein can be extracted using various methods such as accelerated solvent extraction (Kang *et al*., 2016), non-toxic green solvent (Kashyap *et al*., 2022), microwave and enzyme co-assisted aqueous two-phase extraction from marigold (Fu *et al*., 2018), and ultrasound-assisted extraction (Song *et al*., 2018). Kang *et al*. (2016) optimized the extraction conditions for lutein from paprika leaves using accelerated solvent extraction. The parameters tested include temperature of 60, 90, 120, 150 and 180°C, static time of 1, 2, 3, 4 and 5 min, and ethanol concentration of 60, 70, 80, 90 and 100%. The optimal conditions were found to be 93.26°C, 5 min of extraction time, and ethanol concentration of 79.63%. Under these conditions, the lutein yield was 232.60 ug/g. Fu *et al*. (2018) investigated the extraction of lutein and polyphenols from marigold flowers using various methods, including Soxhlet extraction (SE), microwave-assisted aqueous two-phase extraction (MAATPE), enzyme-assisted aqueous two-phase extraction (EAATPE), aqueous two-phase extraction (ATPE) and microwave and enzyme co-assisted aqueous two-phase extraction (MEAATPE). For the MEAATPE method, the optimal parameters were as follows: 28% ethanol, 20% (w/w) ammonium sulfate, 0.45 U/g enzyme concentration, 150 min of enzymolysis, 45°C of enzymolysis temperature, 120 s of microwave time, and 270 W of microwave power. Under this condition, the total polyphenol and lutein yields were 84.61 and 7.32 mg/g, respectively. They found that MEAATPE was an effective and rapid method for extracting bioactive compounds from plants.

The microemulsion extraction technique is a non-

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hazardous and environmentally friendly technique that generally consists of oil, surfactant, water, and sometimes a cosurfactant (Degot *et al*., 2021). Recently, microemulsion have demonstrated the ability to extract various chemical and bioactive molecules, such as enzymes and phenolic compounds (Materna and Szymanowski, 2002; Chatzilazarou *et al*., 2010), lycopene (Jin *et al*., 2008; Papaioannou and Karabelas, 2012), curcumin (Degot *et al*., 2021), lutein (Jalali-Jivan and Abbasi, 2019; Jalali-Jivan *et al*., 2019; Jalali-Jivan and Abbasi, 2020). Surfactant-free microemulsions (SFMEs) are polar microemulsions composed of water and non-polar oil components. A necessary element for the formation of SFMEs is a hydrotrope or amphiphilic solvent (Wang *et al*., 2022). Jalali-Jivan and Abbasi (2020) studied the extraction efficiency of pseudo-ternary mixtures for lutein extraction. They found that using lecithin:1-propanol: water: sunflower with five-cycle extraction significantly increased extraction efficiency by 91–120%, depending on the marigold petals powder ratio. Moreover, the obtained extract exhibited a high DPPH radical scavenging and total antioxidant activity, which were 0.80 mmol TE/g MPP and 14.44 mmol TE/g MPP, respectively. Liu *et al*. (2023) studied the optimum conditions for lutein extraction from marigold petals using microemulsions. They found that the optimum microemulsion solvent was Tween80/n-propanol/isoamyl acetate/water at the ratio of 36:36:18:10 (wt%), which gave the highest extraction efficiency of 95.17%. Degot *et al*. (2021) investigated the SFMEs composed of water/ethanol/triacetin and water/diacetin/triacetin for curcumin extraction. They found that curcumin was soluble in binary mixtures of ethanol/triacetin or diacetin/triacetin. Moreover, the ternary mixture of triacetin/ethanol/water resulted in the highest curcumin extraction yield. Liu *et al*. (2021) studied the solubilization, stability and antioxidant activity of curcumin in SFME containing ethyl benzoate, ethanol, and water. Meanwhile, Russamee *et al*. (2023) studied the binary phase of Triacetin and ethanol for lutein extraction and found that a 4:2 ratio for Triacetin and ethanol resulted in high lutein content with strong antioxidant activity.

Nowadays, there are no reports on the use of the SFME systems for lutein extraction from marigold petals. Moreover, the other compounds, such as phenolic and flavonoid content in the SFME extract, have not been previously reported. Therefore, this work is aimed to investigate the effect of SFME systems on lutein extraction from marigold petals. In the system, triacetin was used as a non-polar component, ethanol served as the hydrotropic solvent and water completed the ternary system. The extracts were determined for the lutein content, as well as total phenolic and flavonoid contents. Furthermore, the antioxidant activity of the extracts was analyzed. The best SFME system was identified using the Fuzzy Analytical Method.

2. Materials and methods

2.1 Materials

The petals of marigold (*Tagetes erecta* L.) were obtained from the Muang District, Nong Khai Province. The clean petals were cut with scissors and dried at 60°C for 24 h. The dried marigold petals were stored in an aluminum foil bag and kept in a dark place for further analysis.

2.2 Ternary phase composition on lutein extraction

Dried marigold petals were extracted following the procedure described by Degot *et al*. (2021) with slight modifications. One gram of dried marigold petals was mixed with distilled water at concentrations of 0%, 2%, 4%, 6%, 8% and 10%. These samples were incubated for 5 min before the addition of a binary solvent mixture of ethanol:TriA (4:2 v/v), using a solid-liquid ratio of 1:25 (w/v) (Russamee *et al*., 2023). The mixtures were then shaken at 200 rpm for 1 h. Supernatants were collected and stored at 4°C. Each ternary phase ratio was repeated three times before further analysis.

2.3 Lutein content determination

The lutein content in the crude extract was determined according to Jalali-Jivan and Abbasi (2020). The lutein content in the crude extract was determined using a UV-visible spectrophotometer at 446 nm and was calculated using an extinction coefficient of 14.45×10^4 L mol^{-1} cm^{-1} and expressed as mg/100 g of marigold petals.

2.4 Total phenolic content

The total phenolic content was determined according to İşçimen and Hayta (2021) with minor adjustments. The 0.2 mL of extract was added to 5 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent, and the mixture was allowed to stand for 5 min. Then, 1.5 mL of sodium carbonate (75 g/L) was added, and the mixture was left to stand at room temperature for 90 min in the dark. At 725 nm, the mixture's absorbance was measured. The Gallic acid standard curve was used to compare the value.

2.5 Total flavonoid content

The total flavonoid content was assessed using the methodology described by Lasunon, Phonkerd, Tettawong *et al*. (2022) with minor adjustments. Distilled water (2 mL) and sodium nitrite (0.15 mL) were added to the 0.3 mL of extract. After 5 min, the mixture was added with 0.15 mL of 10% aluminum nitrate, and it was left for 5 min. An aliquot (1 mL) of sodium hydroxide (1 M) was then added. The mixture's absorbance at 420 nm was measured. The Quercetin standard curve was used to compare the value.

2.6 Antioxidant activity DPPH radical scavenging activity

The antioxidant activities of the crude extracts were determined using two different assays, the DPPH radical scavenging activity and Fe^{2+}chelating activity, according to Russamee *et al*. (2023). Briefly, the 2900 µL of 0.1 mM of DPPH was mixed with the 100 µL of the extracts. The mixture was then left to stand for 30 min in the dark. The DPPH radical scavenging activity was calculated according to the following equation: Scavenging activity (%) = $[(A_s-A_0) / A_s] \times 100$, where A_s was the absorbance of the extract at 517 nm and A_0 was the absorbance of the blank at 517 nm (95% ethanol).

The Fe^{2+}-chelating activity of crude extract was expressed as % Fe-chelating activity compared to the control sample (ethanol). Briefly, the 50 µL of 2 mM $FeCl_2$ was mixed with the 400 µL of extract. The mixture was then left to stand in the dark for 30 min. Then, 200 µL of ferrozine and 3350 µL of ethanol were added to the mixture, and then it was left to stand for 10 min in the dark. The absorbance of the mixture at 542 nm was recorded.

2.7 Fourier transform infrared spectroscopy analysis

The lutein extraction was analyzed using Fourier Transform Infrared Spectroscopy (FTIR). The spectra of ethanol, Triacetrin, best ternary, and lutein standard were recorded using FTIR spectrometer (Bruker Co., INVENIO S, Germany), equipped with an attenuated total reflectance (ATR) unit with wavelengths ranging from 500-4000 nm (Embaby *et al*., 2022). Approximately 10-15 µL of the sample was dropped onto the ATR crystal. After each sample scanning, the crystal surface was carefully cleaned with ethanol and dried with a tissue.

2.6 Statistical analysis

The experiment was planned using a completely randomized design, and the mean differences were observed using Duncan's methods.

2.7 Fuzzy assessment method

The best ternary phase mixture was evaluated using a fuzzy analytical method according to Tongkham *et al*. (2017) and Lasunon, Phonkerd, Pariwat *et al*. (2022), using 5 criteria, including the lutein, phenolic and flavonoid content, DPPH radical scavenging activity and Fe^{2+}-chelating activity value. For each extraction condition, every criterion or experimental result was assessed as a performance score ranging from 0 to 10, based on the minimum and maximum values observed for each criterion. The performance scores were subsequently transformed into a fuzzy performance grade matrix using the triangular fuzzy number. After that, the fuzzy performance grade matrix, combined with relative weights assigned to each criterion, was used to compute the overall performance index. The total weight of all criteria was 100. For this work, the criteria were divided into two main categories: quantity criteria and quality criteria. For quantity criteria, the main bioactive compound was lutein, followed by phenolic and flavonoids. Meanwhile, the quality criteria included two measures of antioxidant activity. Therefore, the assigned weights for each criterion were as follows: 40 for the lutein content, 10 for phenolic content, 10 for flavonoid content, 25 for DPPH radical scavenging activity and 15 for Fe^{2+}-chelating activity.

3. Results and discussion

3.1 Lutein content of the ternary phase extracts

In previous work by Russamee *et al*. (2023), it was found that the most suitable binary phase solvent was a mixture of ethanol and Triacetin in a 4:2 ratio, which effectively extracted high levels of bioactive compounds and exhibited high bioactivity. However, the microemulsions are dispersions consisting of water, oil, and surfactants that are isotropic and thermodynamically stable systems with 1 to 100 nm of dispersed domain diameter. Appearance of microemulsion is clear, formation of self-assembly, long shelf life, low viscosity, and low cost (Kale and Deore, 2017). Surfactant-free microemulsions (SFME) are microemulsions composed of water (polar component) and oil (non-polar component) without surfactant. A microemulsion system has been reported to be ability to extract various chemical and bioactive molecules. For example, Jalali-Jivan and Abbasi (2020) found that a pseudo-ternary mixture of lecithin, 1-propanol, water, and sunflower oil was effective for lutein extraction from marigold petal powder. Russamee *et al*. (2023) found that the binary mixture of triacetin and ethanol at a 4:2 ratio resulted in high lutein content and significant antioxidant activity. When mixing with water, the characteristics of the mixture were consistent with those of microemulsions, clear, stable, and non-separating, exhibiting self-assembly, as shown in Figure 1. Moreover, the ternary phase solvent exhibited long shelf life and low viscosity (1.1440 cP.). These properties confirm that the ternary phase solvent was a surfactant-free microemulsion.

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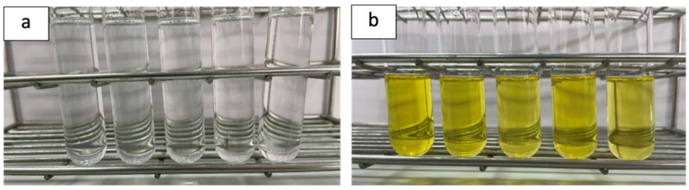

Figure 1. Ternary phase system: a) ternary phase before extraction and b) ternary phase after extraction.

The SFME system has been reported to enhance curcumin extraction, and the addition of water to the binary mixtures of EtOH/TriA can further increase the extraction yield (Degot *et al*., 2021). Therefore, the effect of water concentration on lutein extraction was investigated. The results are shown in Figure 2.

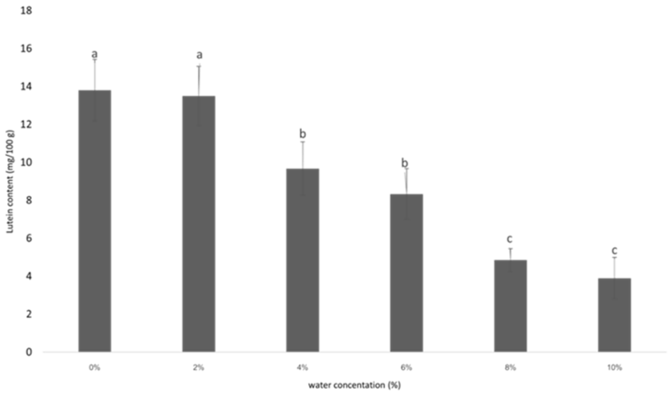

Figure 2. Lutein content of crude extract. Bars with different notations are statistically significantly different (p<0.05).

As shown in Figure 2, when TriA and ethanol were mixed in a 4:2 ratio with a solid-to-liquid ratio of 1:25, lutein could be effectively extracted from marigold. However, it was found that the lutein content gradually decreased as the water concentration increased. The results showed that the water content influenced the extraction efficiency. This may be due to the structure of lutein, which contains conjugated bonds and exhibits hydrophobic properties (Degot *et al*., 2021). At the optimal points, the extractability of the microemulsion reaches its highest level, which means hydrotopes were fully utilized. Subsequently, the further addition of water resulted in a decrease in the extraction efficiency, which may be due to the hydrophobicity of lutein. These results align with previous studies reported in the literature. Liu *et al*. (2023) extracted lutein from marigold using microemulsion systems consisting of Tween 80 (surfactant), n-Propanol (co-surfactant) and isoamyl acetate (oil phase). They found that the extraction efficiency decreased with a high amount of water. Moreover, the addition of 10 % water resulted in the highest extraction capacity; however, extraction efficiency decreased when water concentration exceeded 10%. It was also found that the lutein content of the 2% water concentration was not significantly different from that of 0% water concentration. This might be due to the composition of the emulsion, in this work TriA was used as oil phase and ethanol served as hydrotope in a ratio of 4:2. Nevertheless, the highest lutein content achieved was 13.49 mg/100 g MPP which was lower than the value reported by Jalali-Jivan and Abbasi (2020) who used the microemulsion composed of 50:25:5:20 w/w% lecithin:1-propanol: water and sunflower oil. This discrepancy may be attributed to the differences in oil phase composition and also the variety of raw materials used.

3.2 Total phenolic and total flavonoid content

Marigold petals contain important bioactive compounds, for example, lutein, phenolics and flavonoids. Fu *et al*. (2018) studied the extraction of total polyphenols and lutein from marigold flowers using microwave and enzyme co-assisted aqueous two-phase extraction (ATPE). In their study, ethanol was used as a cosolvent, given its efficiency in extracting phenolics and flavonoids. Regarding lutein extraction, the ternary phase with varying water addition ratios resulted in a decrease in the lutein content. Conversely, adding water to the binary phase solvent may enhance the extraction of polar or water-soluble substances. However, there is currently no research reporting the phenolic and flavonoid content in the microemulsion extracts. Therefore, in our study, the total phenolic and total flavonoid content in ternary phase extracts were determined. The results are shown in Figure 3.

Figure 3a shows that the highest phenolic content

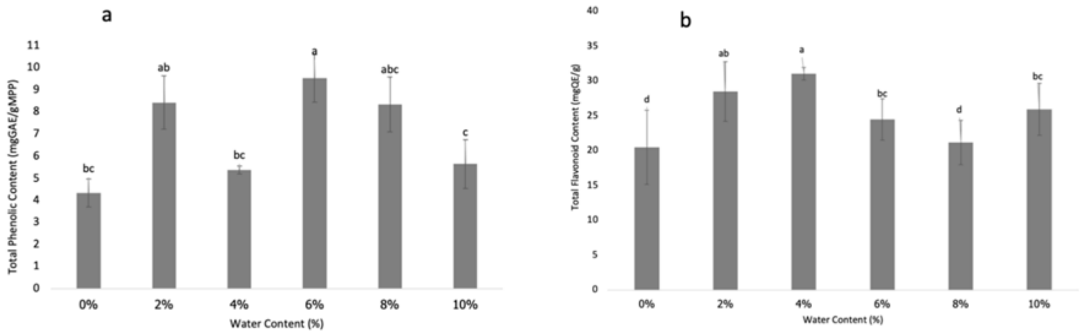

Figure 3. The phenolic content (a) and flavonoid content (b) of crude extract by ternary phase. Bars with different notations are statistically significantly different (p<0.05).

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was found in the ternary phase with 2% and 6% water concentration. This is probably due to phenolics being polar substances, making them more soluble in water, which facilitates their extraction. Similarly, the highest flavonoid content was found at 4% water concentration (Figure 3b). This result might be explained by the polarity and solubility characteristics of flavonoids. However, when the water concentration was further increased, no additional increase in extracted substances was observed. This may be due to the limitation imposed by the polarity of both the solvent and the solutes. However, the maximum total phenolic and flavonoid contents were 9.52 mg GAE/g MPP and 30.98 mg QE/g MPP at water additions of 6% and 4%, respectively. The total phenolic content in this study was less than the finding of Fu *et al*. (2018), who used the microwave and enzyme co-assisted with aqueous two-phase extraction for the extraction of phenolic from marigold, which was 84.61 mg/g. This probably may be due to the differences in the polarity of solvent systems and also the extraction techniques employed.

3.3 Antioxidant activity

Lutein, phenolic and flavonoid are bioactive compounds that exhibit antioxidant activity and are found in the marigolds. The difference in extraction conditions may influence the bioactive compound content in the extract. The binary phase mixed with water in different ratios affected the bioactive compounds in the extract, leading to a decrease in their concentration. This may also contribute to a reduction in the radical scavenging activity of the extract. The DPPH radical scavenging activity and Fe^{2+}-chelating activity of the extract were determined. The results are shown in Figure 4.

The results (Figure 4a) showed that the DPPH radical scavenging activity of extracts did not differ significantly. This result was not correlated with the amount of bioactive compound content. Especially, a decrease in lutein content (Figure 2) and an increase in the phenolic and flavonoid content (Figure 3) were found when the water concentration in the binary phase system increased. Concerning the Fe^{2+}chelating activity, the 2% and 6% water content of ternary phase extracts showed the highest Fe^{2+}chelating activity (Figure 4b). Additionally, the antioxidant activity of the ternary phase extract was higher than that of the binary phase extract. These results may be influenced by the amount and profile of phenolic and flavonoid content. Moreover, the correlation coefficient of bioactive compounds and Fe^{2+}chelating activity was 0.42 and 0.15 for total phenolic and flavonoid content, respectively. Moreover, the Fe^{2+}chelating activity was negatively correlated with lutein content, with a correlation coefficient of -0.40.

3.4 Fuzzy analytical method from the ternary phase

The extraction of lutein using a ternary phase revealed that phenolic and flavonoids were associated with antioxidant activity. Therefore, the phenolic and flavonoid contents were included in the calculation. The fuzzy analytical method was performed to evaluate the best condition for ternary phase extraction. All data criteria were calculated into a performance score ranging from 0 to 10 by using the lowest and highest value of each criterion. Subsequently, the performance scores were converted into a fuzzy performance grade set. The criteria weights for lutein, phenolic and flavonoid content, DPPH radical scavenging activity and Fe^{2+}-chelating activity were set as 40, 10, 10, 25 and 15, respectively. The overall Performance Index of each condition is shown in Figure 5. The result indicated that ternary phase extraction with 2% water concentration achieved the highest overall index.

3.5 FTIR analysis of ternary phase extraction

The FTIR spectrum of all solvents and the ternary extract was determined. The spectrums are shown in Figure 6. The results revealed that the prominent peaks of ethanol were detected at 1045, 1088 cm^{-1}, representing C−O, at 3333 cm^{-1}, representing −OH of the hydroxyl group. A prominent peak of triacetin was detected at 1737 cm^{-1}, illustrating C=O, and at 1370,

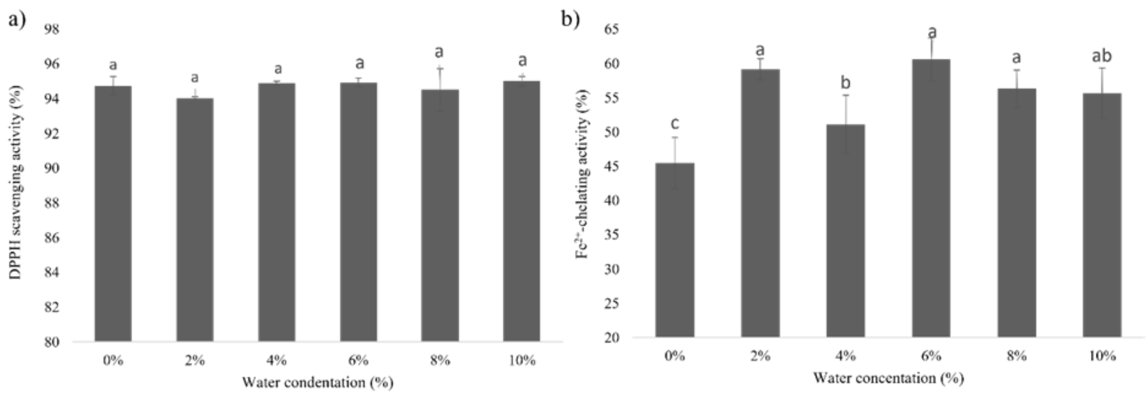

Figure 4. Total antioxidant activity of ternary phase (a) DPPH scavenging activity (b) Fe^{2+}-chelating. Bars with different notations are statistically significantly different ($p<0.05$).

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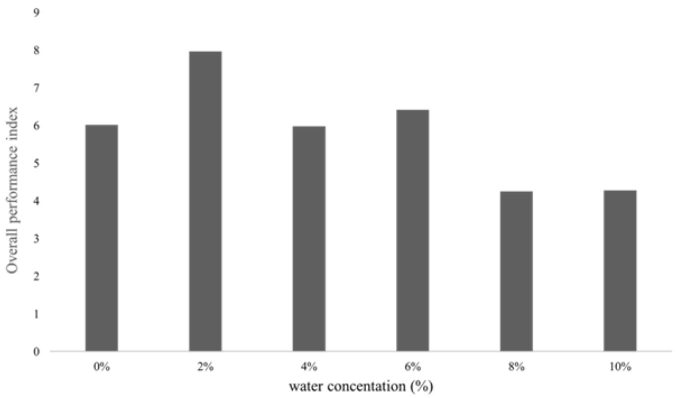

Figure 5. The overall performance index of all crude extracted.

1211 cm^{-1}, illustrating C−H. Moreover, a prominent peak of lutein was detected at 1362 and 961 cm^{-1}, representing dimethyl group and trans conjugated alkene (−CH═CH−), respectively (Yuan *et al*., 2019). Furthermore, the infrared spectrum of the ternary phase solvent also showed the prominent peaks of triacetin (1737, 1370 and 1211 cm^{-1}) with less signal intensity due to the lower amount of TriA in the ternary phase solvent. Regarding the spectrum of extract obtained using binary phase extraction, a prominent peak of lutein was detected at 960 cm^{-1}, confirming that the lutein was successfully extracted. Moreover, the prominent peak of triacetin (1737, 1370 and 1211 cm^{-1}) was also found. This indicates that the ternary phase system, or surfactant-free microemulsion system, has a significant effect on lutein extraction.

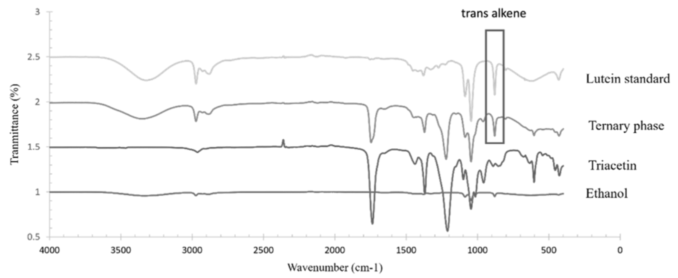

Figure 6. FTIR spectrum of lutein extracted.

4. Conclusion

The results showed that upon the addition of water to a binary phase mixture containing ethanol and TriA, a decrease in lutein solubility was found with increasing water content. The addition of water to the extraction systems caused an improved extraction power of other substances, such as phenolics and flavonoids, from marigold petals, thereby improving the extraction of polar substances. Moreover, the antioxidant activity of the SFME extract was higher than that of the binary phase extract. The Fuzzy Analytical Method indicated that the ternary mixtures with 2% water concentration were the most suitable green solvent for lutein extract from marigold petals. These findings confirm that the SFME can be used as an innovative and eco-friendly solvent for lutein extraction from marigold petals.

Conflict of interest

The authors declare no conflict of interest.

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