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BIOCHEMICAL CHARACTERIZATION AND *IN VITRO* RELEASE STUDY OF ALGINATE MICROPARTICLES PREPARED WITH INDIAN ROSE (*ROSA GRANDIFLORA*) FLOWER PIGMENTS

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INTRODUCTION

The economic implications of polyphenolic compounds are vast. Since consumers' interests are shifting towards healthy and minimally processed foods, synthetic antioxidants are slowly losing ground in their application in commercial products, although they are much stable and can withstand circumstantial onslaughts. Polyphenols in the form of natural antioxidants thus gained immense importance as commercial means. They are used in various sectors of the food-processing industry as natural colouring agents, preservative agents, natural antioxidants or nutritional enhancers.[1] However, it is probably in the field of human health that the commercial application of polyphenols is the most important.[2] Actually, many plant extracts rich in phenolic compounds of interest are used not only as food complements, they can be integrated into cosmetic or pharmaceutical formulations also. Unfortunately, the use of these important compounds are substantially limited for human application. Although these compounds showed excellent activities in *in vitro* models, their efficacies reduced when applied *in vivo*.[3] Moreover, route of administration plays a crucial role in their bioavailability and integrity in experimental models. Orally administered polyphenols may have insufficient gastric residence time, low permeability and/or low solubility due to their different behaviour towards different conditions like pH, enzymes or presence of other nutrients that may limit the activity and their potential health benefits. Furthermore, many polyphenols in their free form may show limited to no water solubility, susceptibility to

environmental oxidation, or they might have disagreeable tastes, which must be concealed before their integration in foodstuffs or oral medicines.

A number of techniques have been evolved over years for efficient systemic delivery of functionally important natural substances, amongst which, microparticles, microspheres, and microcapsules are common and collectively called as multiparticulate drug delivery systems.[4] These particles differ in their internal structure or size and composition of matrix.[5] The technology is known as 'Microencapsulation' which basically is the formation of a protective structure (viz. wall material) covering a substance of interest (viz. core material) for its protection and controlled release.[6] These microencapsulated products are widely used not only in the food, pharmaceutical and cosmetic industries, but also in various other domains like personal care, agriculture, veterinary medicine, industrial chemicals, biotechnology, biomedicines and biosensor development sectors.[7] These bioactive microparticles may contain a solid, liquid or gaseous active substances having size that can range from sub-micron level to about a few mm.[8]

Lots of studies have been emerged out in recent past showing effectiveness as well as acceptance of antioxidant-rich health drinks devoid of synthetic pigments or sweeteners.[9] Rose flower is one such antioxidant-rich source that is also well known for its edible nature and has been consumed for a long time in teas, cakes, and flavour extracts, as well as being a traditional remedy to treat blood circulation related complications and to control cancer growth.[10] There is a worldwide trend of using edible roses as not only an ingredient in various foods, but also as raw material for anti-inflammatory drugs due to their efficacy for inhibiting histamine release.[11] All these bioactivities are attributed to the rich anthocyanin contents of the petals of rose flower.[12]

Anthocyanins, a family of positively charged flavonoids, are responsible for the brilliant colours of numerous fruits, flowers and vegetables and their specific colour depends on co-pigments, metal ions and pH.[13] However, anthocyanins have a major disadvantage regarding their stability that is influenced by a number of factors like pH, light, temperature, co-pigmentation, sulfites, ascorbic acid, oxygen and enzymes.[14] A previous study from our laboratory established development of a delivery system by synthesizing microparticles made up with alginate and pectin, using ionotropic gelation method, for effective protection and delivery of polyphenols isolated from peels of orange (*Citrus aurantium*) and sweet lime (*Citrus limetta*).[15] In perpetuation to that perception, the objective of our present study was

to prepare microparticles of anthocyanin-rich extracts of rose flower to adjudicate the possible controlled release of the antioxidative component at different pH *in vitro*. This would help in understanding the stabilities of the antioxidative components in physiological conditions, if applied systemically. Biochemical characterization of the microparticles was also done using some *in vitro* antioxidant analyses.

MATERIALS AND METHODS

Materials

Fresh rose flowers of three different colours were obtained from local market, washed thoroughly to remove soil and dirt, partially dried by pressing between cheese cloths, and used on the same day for extraction. The flowers were checked for visible damage and those flowers were rejected. Sodium alginate (food grade, conforming to NF) and pectin (pure) were purchased from Loba Chemie, India. All other chemicals were of AR grade and purchased from Merck, India or SRL, India. All the spectrophotometric determinations were done in a Systronics (India) double beam UV-Vis spectrophotometer (model – 2202). Double distilled water was used in all the experiments.

Extraction of rose-petal

Three different colour variants of rose flower were used in this study – red, pink and orange. Aqueous fraction of the rose petals were extracted using water as solvent. Briefly, 1 gm of fresh rose petal was heated at low-flame with 100 ml water for 10 minutes with occasional stirring. The mixture will then be filtered with Whatman#1 filter paper and stored at 4-6^{o}C for microparticle preparation.

Preparation of Microparticles

Microparticles were prepared by ionotropic gelation method using two independent variables – sodium alginate and pectin.[15] Firstly, pectin (2.5%, 2.5 g in 100 ml) and sodium alginate (5%, 5 g in 100 ml) were dissolved in water and the rose petal extract from three different varieties were dispersed in it at volume ratios of 1:1, separately. Separately, the mixtures were then added drop wise through an injection needle on a gently agitated $CaCl_2$ solution (0.1 M, 1.1 g in 100 ml). Subsequent microparticles obtained by the gelation of the pectin and the alginate were held in magnetic stirring for 3 hours. The particles were then separated, washed with distilled water and dried *in vacuo*. Microparticles prepared from red rose was designated as – M_R, from pink rose was designated as – M_P and from orange rose was

designated as – M_O. Control microparticles were also prepared devoid of rose petal extracts and designated as – M_C.

Estimation of monomeric anthocyanin contents

Determination of total monomeric anthocyanin content in the microparticles before stability studies was done following a published method.[16] Total monomeric anthocyanins were expressed as cyanidin-3-glucoside. Sample absorbance was read against a blank cell at 700 nm and 510 nm and at pH 1.0 and 4.5. The absorbance (A) of the sample was then calculated according to the following formula

$$A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$$

Where A is the net absorbance of samples at the wavelengths mentioned in the subscript. The monomeric anthocyanin pigment contents in the experimental solutions were calculated according to the following formula

$$\text{Anthocyanin content (mg/l)} = (A \times MW \times DF \times 1000) / (\varepsilon \times l)$$

Where DF was dilution factor, MW was molecular weight of cyanidin-3-glucoside (449.2), l is the path length and ε was molar absorptivity (26,900). The anthocyanin content was expressed as mmol / gm microparticle.

Stability of the Microparticles

A preliminary stability test for the microparticles were performed in solid condition for 21 days. The microparticles were placed in room temperature (28±2^{o}C), both at dark and light conditions, separately, and at refrigerator at 6±2^{o}C and analysed for their polyphenol contents using Folin-Ciocalteau method at 0th, 7th, 14th and 21st days.

Release of loaded polyphenols at different pH conditions

The content of extract released from weighed amounts of microparticles was determined by soaking a known amount of capsule in the release media at 37^{o}C while agitating in an Orbit-Environ Shaker at 125 rpm.[15] The release media used were *in vitro* model digestion fluids, i.e. Simulated Gastric Fluid, SGF (pH 1.6) and Simulated Intestinal Fluid, SIF (pH 7.2). SGF was produced by introducing NaCl (2.0 g) into 0.1M HCl (1.0 liter), and SIF was made from a mixture of 0.2M $Na_3PO_4 \cdot 12H_2O$ (200 ml) and the SGF (800 ml) at a volume ratio of 1:3.[17] A simulated alkaline release media was also prepared with sodium carbonate-bicarbonate

buffer (pH 9.2). The amount of extract released after a stipulated time period was determined as polyphenol content.

Estimation of polyphenol content

Polyphenolic compound contents were determined by the classical Folin-Ciocalteau method, as described elsewhere.[18] Briefly, the samples/standards were mixed with Folin-Ciocalteau reagent (1:10 diluted with distilled water) for 5 min and aqueous sodium carbonate (1 M) was then added. The absorbance of the reaction mixture was then measured at 765 nm. Gallic acid was used as standard. The results were expressed in terms of gallic acid equivalent/gm microparticle.

Statistical analyses

Experimental results were expressed as mean of four individual experiments. Analyses were done using the software "PSPP ver 1.6.2" (GNU Project).

RESULTS AND DISCUSSION

Pectin and alginate have been widely used in food applications for preparation of microparticles because of their biocompatibility, biodegradability, non-toxicity and low cost. Preparing antioxidant-loaded microparticles by these components generally accounted for the highest encapsulation efficiency among other methods.[19] Pectin is a natural anionic, linear water-soluble polysaccharide consisting of galacturonic acid, whereas alginate consists of two monomeric sugar units viz. β-D-mannuronic acid and α-L-guluronic acid. Moreover, alginate-pectin mixtures might have synergistic properties that allow them to form different microstructure during formation of biopolymers. In the present study, extrusion technique for microparticle preparation was used due to its simplicity and low cost of production.[20] Fig.1 showed the three microparticles prepared with three different rose flower extracts. A control microparticle was also prepared without any pigment for comparative evaluation.

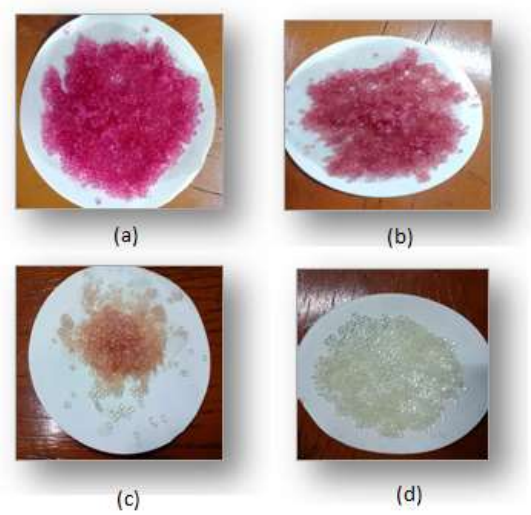

Figure 1: Microparticles prepared with three colour varieties of rose flower pigments viz. (a) Red (M_R) (b) Pink (M_P) (c) Orange (M_O) and (d) without any pigment (M_C) using sodium alginate and pectin.

After preparation of the microparticle beads, anthocyanin contents were determined afresh. Results are indicated in Fig. 2.

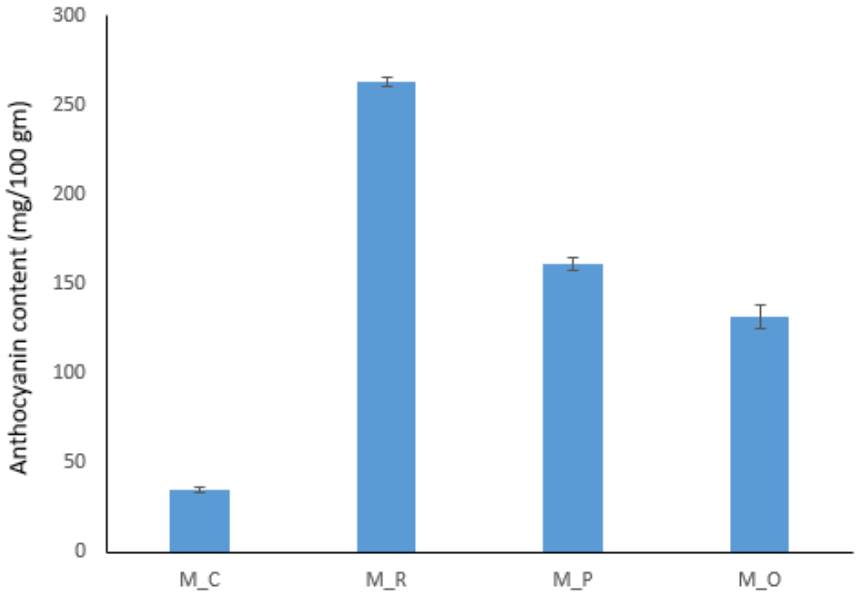

Figure 2: Anthocyanin content of the microparticles prepared with three colour varieties of rose flower pigments using sodium alginate and pectin. Results are mean of four individual observations ± standard error of mean (SEM).

The anthocyanin content in the microparticles were in accordance with established facts that red or different tones of red colour in flowers are due to presence of anthocyanins where the

intensities of the colours vary primarily due to differential expression of anthocyanin producing genes or the pH of the environment.[21] Even different classes of anthocyanins are responsible for different colour tones. Generally, pelargonidin and cyanidin cause petals to produce red and purple colors, respectively, while delphinidin produce blue and purple colors.[22] In the flower variants with less intense red colour, the contents of some flavanols and flavonoids, such as kaempferol, quercetin, apigenin, and luteolin becomes more prominent.[23] That is why, dark colours of anthocyanin in the microparticles tended to be less in those prepared with pink or orange flower extracts as was evident from both Fig. 1 and Fig. 2.

The stability of the microparticles was influenced by the differential contents of the pigments as was observed when the particles were put in three different storage conditions and contents of polyphenols were estimated after stipulated time periods. In case of microparticles prepared with red rose extracts, a sustained stability was observed up to 21 days if the samples were kept at dark but at room temperature (Fig. 3). However, in case of pink rose extracts, the microparticles were more stable at refrigerator (Fig. 4). In case of microparticles prepared with orange rose pigments, the stability was not so prominent at any of the experimental conditions. (Fig. 5).

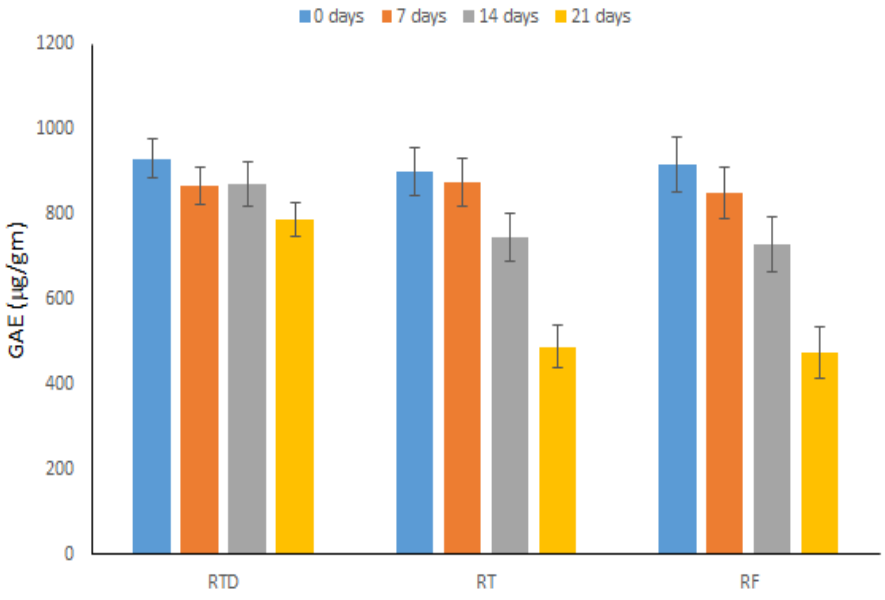

Figure 3: Stability of the microparticles for 21 days at different conditions, prepared with flower pigments from red rose. Results are mean of four individual observations ± standard error of mean (SEM).

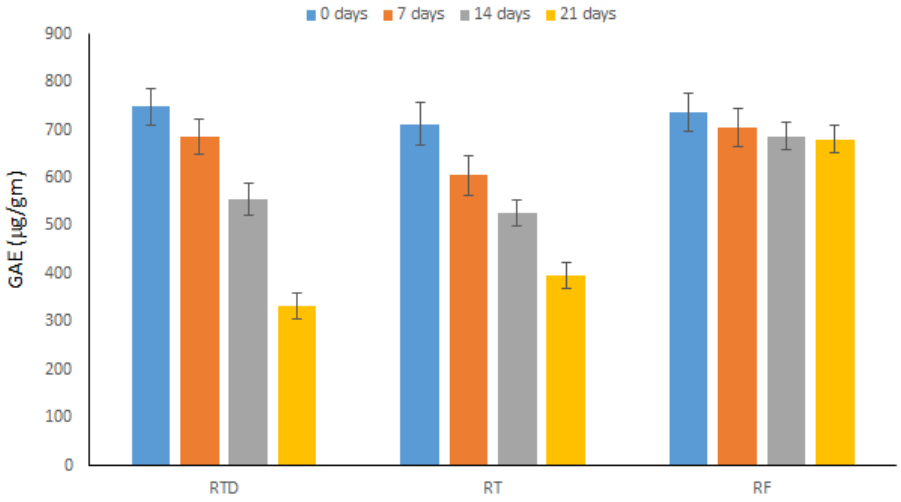

Figure 4: Stability of the microparticles for 21 days at different conditions, prepared with flower pigments from pink rose. Results are mean of four individual observations ± standard error of mean (SEM).

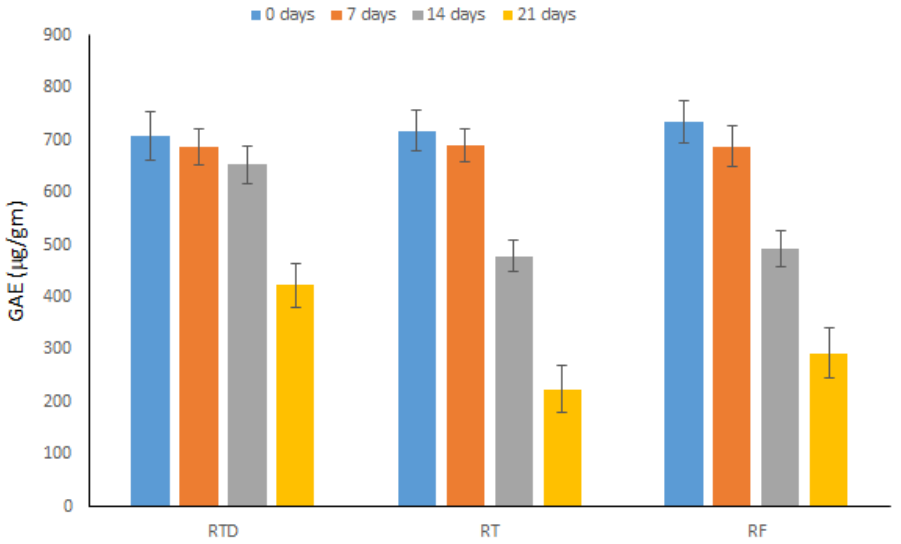

Figure 5: Stability of the microparticles for 21 days at different conditions, prepared with flower pigments from orange rose. Results are mean of four individual observations ± standard error of mean (SEM).

Copigmentation is a natural process based on noncovalent complexation of biomolecules, especially with certain colors like blue, violet, and red.[24] The process plays an important role in stabilizing colours in flowers, vegetables, and fruits, as well as in food products derived from them. Presence of extended π-conjugated systems in anthocyanins allow them to absorb in the visible range and they readily form supramolecular assemblies with other pigments and

cofactors like phenolics and flavonoids. One way of stabilizing color during food processing and storage is to add specific biomolecules as copigments, a technique intensively used for preparation of berry juices.[25] Phenolic acids were being most important to enhance color stability in some berry juices when added during storage.[26] The present study indicated that microparticles prepared from red and orange roses were appreciably stable at room temperature when kept in dark up to 14 days. When kept at room temperature only in presence of ambient light, their stabilities were not so prominent. Since phenolics are one most prominent component of the rose petal compounds,[27] they might play a positive role in stabilizing the colour at room temperature. One interesting observation was that apart from the microparticles prepared from pink rose extract, the other two showed poor stabilities even at refrigerated conditions. As previous studies showed that pink roses contain high amounts or flavonoids and very low carotenoids,[28] the present observation indicated that copigmentation with flavonoids might play a crucial role in stabilities of the colours when extracted and incorporated in microparticles.

One important objective of the present study was based on the possible effectiveness of release of rose petal pigments loaded in microparticles in different pH conditions that would delve into their activities in the alimentary system of humans. Also the bioavailability of several plant extracts were proved to be affected by changes occurring during the gastrointestinal transit.[29] In the present study, such release patterns were observed in three typical pH conditions – viz. 1.6, 7.2 and 9.2. A steady release of the antioxidants up to 60 minutes at pH 1.6 was observed in the present study. After that, a steady release pattern was followed in the solution. Similar type of steady release was also observed at pH 7.2, although it was up to 30 minutes. However, at pH 9.2, the release was erratic although increase was consistent up to 120 minutes. This might be due to simultaneous degradation of the bead components at high alkaline pH along with the release. This indicated the adversative usefulness of such bead in alkaline physiological conditions. The results are furnished in Figs. 6, 7 and 8.

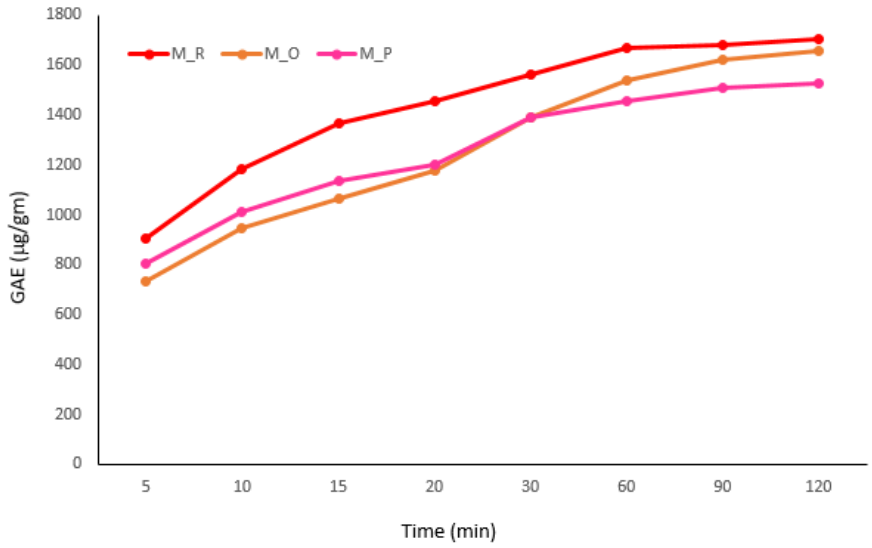

Figure 6: Release of polyphenols from the microparticles prepared with three different rose flower extracts viz. Red (M_R), Pink (M_P) and Orange (M_O), over 120 minutes at pH 1.6. Results are mean of four individual observations.

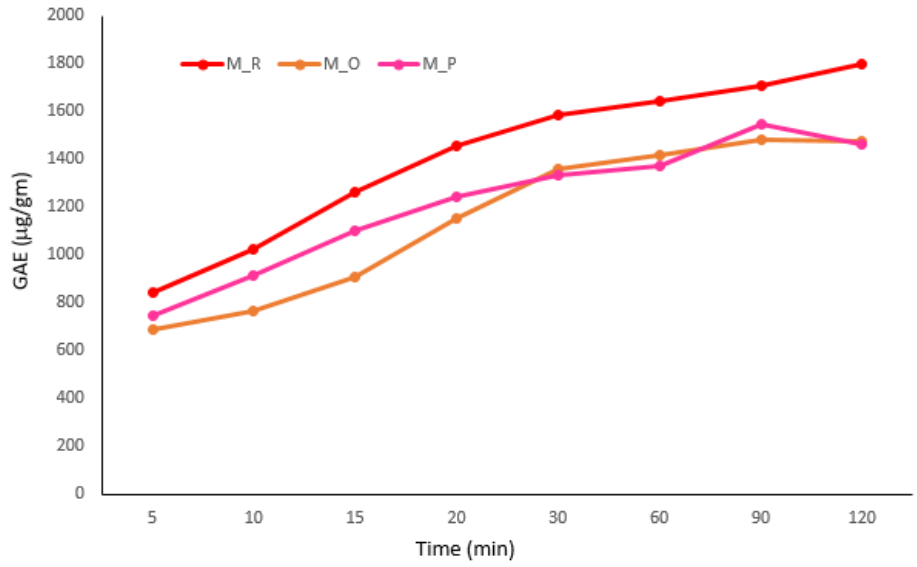

Figure 7: Release of polyphenols from the microparticles prepared with three different rose flower extracts viz. Red (M_R), Pink (M_P) and Orange (M_O), over 120 minutes at pH 7.2. Results are mean of four individual observations.

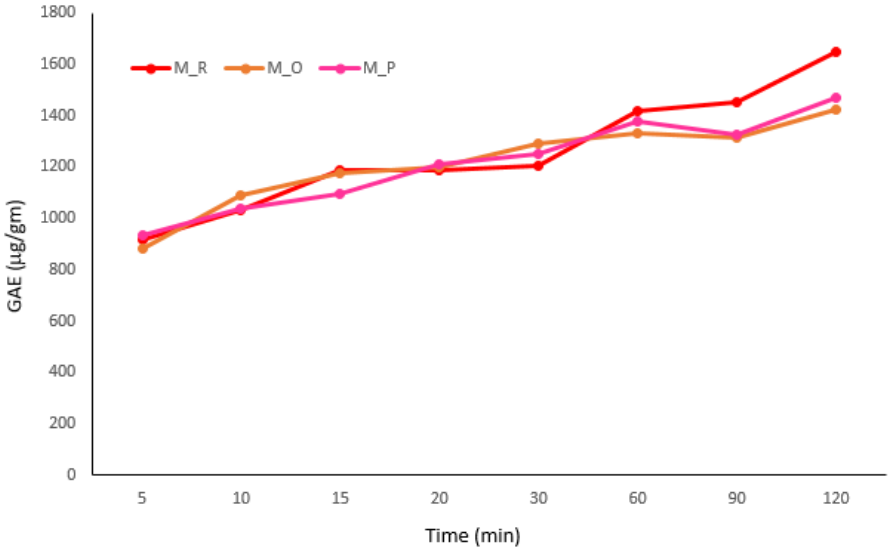

Figure 8: Release of polyphenols from the microparticles prepared with three different rose flower extracts viz. Red (M_R), Pink (M_P) and Orange (M_O), over 120 minutes at pH 9.2. Results are mean of four individual observations.

CONCLUSION

The stabilities of three different microparticles prepared with different rose flower aqueous extracts and formed by ionic gelation method with different combinations of sodium alginate and pectin were promising over a period of 21 days. Presence of other biomolecules in the extracts positively modulated the stability due to copigmentation. It was also observed that copigmentation with flavonoids might play a crucial role in stabilities of the colours when extracted and incorporated in microparticles. The particles released their antioxidative components in simulated physiological fluids in a controlled manner. The microparticles showed excellent results at pH 1.6 when steady release was observed after 60 minutes. Steady release was observed after 30 minutes at pH 7.2 clearly indicated effectiveness of the microparticles in physiological conditions as it might steadily release the components only after 30 minutes of incorporation. Although it was an *in vitro* study, but the methodology, results and the overall success of this study could be the first step towards a signal regarding the probable outcome of their effects when applied *in vivo*. They can be used as food supplements, as nutraceuticals, as drug supplements, or as functional food delivery system in future without any hesitation of unfamiliar non-edible substance ingestion.

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