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Quantification of Selected Bioactive Compounds in Instant Coffee and Their Effect on Gastric Release using HGT-1 Cells

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ABSTRACT

Introduction: This study was carried out to quantify the selected bioactive compounds (i.e., chlorogenic acids, caffeine, and N-methylpyridinium) in instant coffee and to analyze its correlation with the gastric release effect of the HGT-1 cell line. **Methods:** Selected bioactive compounds in regular (REG), low sugar (LS), low fat (LF), white coffee (WC), white coffee low acid (WCA), decaffeinated (DC), and instant black coffee (BC) were quantified using HPLC-DAD (high-performance liquid chromatography diode array detection) system and flow cytometry analysis for its gastric release effect when treated with HGT-1 cell. **Results:** The HPLC data showed the content of caffeine (60,212 ± 212 µg/ml) and chlorogenic acid (35,779 ± 3027 µg/ml) were significantly high in BC while the lowest caffeine value was found in DC coffee. Chlorogenic acid in other instant coffee samples showed insignificant content distinctions. As for N-methylpyridinium (NMP), the highest content was found in BC (565 µg/ml) and the lowest value was detected in WC (52 µg/ml) coffee. Gastric release activity by HGT-1 cells was significantly higher in DC and REG coffee treatment. Pearson correlation showed no significant correlation between the quantitative data and gastric release activity by HGT-1 cells. **Conclusion:** The selected bioactive compounds contained in instant coffees were unable to stimulate gastric release.

Keywords: Caffeine, Chlorogenic acids, NMP, HGT-1, Gastric acid release

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INTRODUCTION

Coffee has a long origin history and was initially introduced as a lifestyle beverage. Over the years, the progress of coffee production had shown a worldwide increment due to its high demand and consumption level (1). The stimulating properties and captivating aroma are among the characteristics heavily appreciated by habitual coffee consumers. Collective research data on coffee had concluded numerous health-benefiting properties such as protection against diabetes mellitus, neurodegenerative disease, cancer, cardiovascular disease, and cataracts (2). However, dyspeptic symptoms linked with gastrointestinal problems have been a common side effect to several avid drinkers. The dyspeptic condition has been defined as a condition where a person encounters recurring pain at the upper abdomen which causes prolong discomfort (3). In relation to coffee, the more common dyspeptic symptom exerted post-drinking is the over-stimulation of gastric acid secretion (4,5). Ironically, customary dietary prohibition for patients with gastrointestinal problems is coffee consumption. In Malaysia, the demographic prevalence of dyspepsia was found higher in the urban area (25%) than in the rural area (15%) (6). The study, however, excluded the discussion of respondent's coffee-drinking habits as a causal factor contributing to the prevalence value generated. Moreover, Malaysians coffee consumption is currently at growth (7), however, congruences between coffee-gastrointestinal-related studies were imbalanced due to an inadequate amount of local research.

Most coffee-related data were concluded from observational studies while others were solely based on myths that were supported by unfounded evidence. Hence, extensive scientific-based evidence is desperately needed for in-depth discoveries on the chemical compounds as well as the interaction between coffee matrix and the mechanisms involved which are still lacking (8). Previously published data on roasted coffee beans subjected to steam treatment were found to be proficient in reducing gastric complications following the reduction of compounds causing gastric discomforts such as caffeine, chlorogenic acid, and C5HTs (N-beta-Alkanoyl-5-hydroxytryptamides) in its content (9,10). In addition, compound such as NMP

(N-methylpyridinium) in coffee was recently discovered to have the ability to downgrade gastric-release rather than stimulating the gastric acid (8,11). Subsequently, these selected bioactive compounds in coffee and their effect on gastrointestinal health have been studied in-depth by numerous researchers to further extract valuable information regarding the matter (11–15). In another study, weak correlations were found between these selected bioactive compounds and their influences on gastric release (11). Most of these generated data incorporated a variation of brewed coffee samples that undergo different technological processes and ingredients for its final product. These technological processes could alter the percentage value of compounds in coffee to the extent of affecting its distinct aroma and taste (16). Consequently, in efforts to overcome the side effect, the addition of supplementary substances was made into the coffee mixtures which require further investigations.

In this study, the quantitative data and gastric effect of selected bioactive compounds (i.e. Chlorogenic acid and its isomers, caffeine, and N-methylpyridinium) in seven categories of instant coffee samples were determined and compared. This study anticipated a positive correlation between the instant coffee samples and the gastric release effect. The conclusion generated from this study will partly redound to the gastrointestinal health of Malaysians and might be able to improve their coffee intake management. Hence, it is essential to establish a resilient coffee-related database that acts as a reliable and comprehensive source of reference to the public.

MATERIALS AND METHODS

Chemicals and Reagents

5-caffeoylquinic acid (5-CQA, 96%), 4-caffeoylquinic acid (4-CQA, 98%), and 3-caffeoylquinic acid (3-CQA, 98%) standards were purchased from Toronto Research Chemicals (Toronto, Canada) as reference materials. N-Methylpyridnium (NMP, 98%) standard was purchased from Sigma-Aldrich (Darmstadt, Germany) as reference materials. Formic acid 98%-100% LC-MS LiChropur (Merck, Darmstadt, Germany), HPLC grade water (Fisher, Massachusetts, USA). Methanol HPLC grade (MeOH) (J.T. Baker) and Acetic Acid HPLC grade (Sigma-Aldrich, St. Louis, MO, USA). Dulbecco's Modified Eagle Medium with 4% glucose (DMEM, Gibco, WA, Massachusetts), accutase (Sigma-Aldrich, Darmstadt, Germany), fetal bovine serum (FCS, Gibco, WA, Massachusetts), penicillin-streptomycin (Gibco, WA, Massachusetts), phosphate buffer saline (PBS, Sigma-Aldrich, St. Louis, MO, USA), 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-buffer (Sigma-Aldrich, St. Louis, MO, USA). Thiazolyl blue formazan (MTT) (Sigma-Aldrich, St. Louis, MO, USA). Ultrapure water was obtained from the Milli-Q water purification system (Millipore, Bedford, MA, USA). The human parietal carcinoma cell line (HGT-1) grown by Dr. C. Laboisse was purchased and obtained at passage 9 from Ephyscience company in Nantes, France.

Coffee sample collection

Different products declaration of commercialized instant coffee was purchased from the local markets. The selection of coffee brands was made based on the published statistical data by Euromonitor (17). The statistical analysis generalized coffee brands and producers with the highest production and local household penetration value. The instant coffee samples were then categorized into 7 different categories according to the product's declaration: regular instant coffee (R, n=3), low sugar instant coffee (LS, n=3), low-fat instant coffee (LF, n=3), instant white coffee (WC, n= 3), low acid instant white coffee (WC-LA, n= 3), decaffeinated instant coffee (DC, n=3) and instant black coffee (BC, n=3) as a control sample. The black coffee does not contain any added ingredients such as preservatives, additives, creamer, and sugar. Hence, black coffee has been used as a control sample to observed the gastric release effect of other coffee samples containing added ingredients.

List of ingredients

Instant coffee has been continuously innovated into other types of coffee products such as 3-in-1 instant coffee by including added ingredients of milk and sugar. The ingredients in the list that might induce gastric release are sugar, creamer (dairy and non-dairy), sodium caseinate, skimmed milk. These ingredients existed mostly in the instant coffee samples and its gastric effect has been compared with black coffee samples. Table I below shows the listed ingredients of coffee products used in this study.

Coffee sample and standard solution preparation

In sample preparation, each coffee stock was mixed as recommended by the manufacturer; 20 g of instant coffee (n= 7) was mixed with 200 ml of boiling water. This is to reflect the nearest possible normal intake of coffee. Furthermore, the temperature of brewing the coffee has no significant effect on the bioactive compounds in coffee (18,19). The stock coffee mixture with 100mg/ml concentration later underwent serial dilution with a diluted final concentration of 3.3mg/ml. The final diluted concentration was filtered through 0.45 mm membrane filters and injected into the HPLC-DAD system. The wavelengths used were 270 nm for caffeine and 320nm for chlorogenic acid quantification. As for N-Methylpyridinium, the stock coffee mixture was diluted into the final coffee concentration of 50mg/ml for each coffee type. The coffee was then filtered through the 0.45 mm membrane filters and injected into the HPLC-DAD system using a wavelength of 260 nm in triplicates.

The caffeine and chlorogenic acid standard solutions were prepared using a volumetric flask and were

Table I: List of ingredients for each type of coffee sample tested in this experiment

Types of coffee	Ingredients
BC	Arabica and Robusta blend
DC	Arabica and Robusta blend
REG	Sugar, creamer, glucose syrup, hydrogenated palm kernel oil, stabilizers, sodium caseinate (milk protein), emulsifiers, anticaking agent, salt, flavorings, instant coffee, skimmed milk powder (cow's milk). All additives are of plant or synthetic origin
LF	Sugar, non-dairy creamer, glucose syrup, hydrogenated vegetable fat, sodium caseinate (milk protein), emulsifier, stabilizer, silicon dioxide, and instant coffee.
LS	Creamer, glucose syrup, hydrogenated palm kernel oil, sodium caseinate (milk protein), salt, stabilizer, emulsifier, anticaking agent, instant coffee, skimmed milk powder (cow's milk), flavoring. All additives are of plant and synthetic origin.
WC	Sugar, creamer (glucose syrup, hydrogenated palm kernel oil, stabilizers, sodium caseinate (milk protein), emulsifiers, flavorings, anticaking agent, salt, maltodextrin, instant coffee, malted milk powder (malt extract-barley), skimmed milk powder (1.3%) (Cow's milk), palm oil. All additives are of plant or synthetic origin.
WCA	Sugar, Non-dairy creamer, glucose syrup, refined hardened vegetable lauric fats (coconut and/or palm kernel oil), sodium caseinate (milk protein), stabilizer, emulsifier, anticaking agent, foaming creamer (glucose syrup solids, vegetable fat, milk solids, stabilizer, emulsifier, flavoring), instant coffee, coffee flavor

The ingredients are listed based on the predominant sequence.

dissolved in methanol following the 1mg: 1ml ratio. Standard compound N-methylpyridinium iodide (95% purity) was dissolved in ultrapure distilled water. The standard solutions underwent serial dilution to make up the concentration range of 1000µg-15.63µg. Each concentration with a final volume of 1 ml was triplicated to establish the standard curve and perform the analyses of linearity, the limit of detection (LOD), and the limit of quantification (LOQ) for the method.

Chromatographic condition

The current study follows Chew and colleagues in performing chromatographic analysis for chlorogenic acid and caffeine with some modifications (20). The analysis was divided into mobile phase A and mobile phase B which uses 0.5% acetic acid (v/v) and methanol, respectively. Gradient elution was used and the profile was: 0–20 min, linear gradient from 0% to 90% B; 20–25 min, 90% B isocratic; 25–30 min, linear gradient from 90% to 0% B, and finally, the column was washed and reconditioned. The column's temperature and flow rate were set at 1.0 mL/min and 30 °C, respectively. Wavelengths used to detect caffeine and phenolic acids were 270nm (caffeine) and 320 nm (chlorogenic acid). The total chlorogenic acid (CGA) was calculated as the sum of the main CGA isomers: 3-CQA, 5-CQA, 4-CQ.

Analysis of phenolic compounds incorporated the HPLC system known as Agilent 1100 Series liquid chromatographic system, equipped with an Agilent 1100 Series diode array detector (DAD) (Agilent Technologies, Germany). The column Phenomenex Luna (5u C18 (2) 100 A. 250 x 4.6mm Phenomenex, United States) was used for separation.

Quantification of NMP compound was made based on Gant and colleagues' with some modifications (19). The chromatographic separation was performed on a Kinetex PFP column (150 Ч 4.6 mm, 2.6 µm; Phenomenex, Torrance, CA). The isocratic elution was used, and the mobile phase was a mixture of two solvents: 95% A (0.05% aqueous formic acid) and 5 % B (100% MeOH) with a post time of 10 minutes, totalling the elution time = 30 minutes. The flow rate was 0.5 mL/min, and the temperature of the column was set at 30°C and monitored at a wavelength of 260 nm. All samples were filtered through a 0.45 µm syringe filter (Thermoline) before analysis and each sample was injected (20 µL) in triplicate. The peak of NMP compound in the coffee samples was identified by comparing retention time between coffee samples spiked with NMP standard and the coffee samples.

Analytical quality control

The quality of the analytical data was carried out according to the criteria proposed by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use for repeatability and reproducibility (21).

HGT-1 cell culture methods

The method used for cell culture was based on previously conducted research (11). The human parietal carcinoma cell line HGT-1 grown in standard tissue culture conditions at a temperature of 370C and 5% CO2 was used to culture the cell. The medium of Dulbecco's modified Eagle's medium-high in glucose (4%) being supplemented with 20% fetal bovine serum, 2% penicillin-streptomycin, and 2% 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) buffer (Sigma-Aldrich, Munich, Germany). The seeding of cells took place in a T75 flask and was cultivated with fetal bovine serum (FBS) for 3-5 days, reaching a confluence of 90-100%. Regarding MTT assay and flow cytometry assay, phosphate-buffered saline (PBS;Sigma-Aldrich, Munich, Germany) was used to wash the cells once which were later detached using accutase (Sigma-Aldrich, Munich, Germany) to obtain the suspended cells.

Secretory activity measurement by flow cytometry

The method developed by Weiss and colleagues (10) was adopted in measuring the secretory activity of HGT-1 cells treated with coffee samples. Each biological independent experiment used an aliquot (2 mL) of the cell suspension corresponding to 2,000,000 cells and was incubated for 10 minutes with 2.5mg of coffee lyophilize per mL of PBS at 370C. Afterwards, a 3 µmol/L concentration of the fluorescent pH-sensitive dye 1,5-carboxy-seminaphtorhodafluor acetoxymethyl ester (SNARF-AM, Invitrogen) was added and the incubation

process was continued on ice for another period of 30 minutes. Lastly, the final step involved centrifuging the cells at 300g for 5 minutes and buffered in ice-cold PBS before being subjected to the flow cytometric measurement.

Flow cytometer condition

The flow cytometer BD FASCANTO II was used to excite the dye with 500 mW of the 488 nm argon laser line. Two fluorescent bands (FL-1=550-590nm, FL-2 600nm) were collected as an integral signal for the calibration curve and sample-treated cells, whereby an analogue function board was utilized to calculate the ratio (FL-2/FL-3). The ratio channel number is linearly related to the actual ratio FL-2/FL-3, with the ratio of 1 being in channel 256. The filter combination to achieve these fluorescent bands was a 550 nm blocking filter, a 590 nm dichroic filter, and a 610 nm long-pass filter. In reference to the validation experiments, the concentration of intracellular pH was calculated and expressed in nmol/L. Meanwhile, the intracellular proton index (IPX) calculation was performed by applying log2 transformation on the intracellular proton concentration ratio between treated cells and control cells for all other experiments.

Calibration curve

Each experiment was subjected to a process of staining the cells in potassium buffers of varying pH values to generate the calibration curve. The pH values, ranging from 6.0, 6.8, 7.2, 7.6, 8.0, and 9.0 was also added with 2 µmol/L of the ionophore nigericin to maintain a state of equilibrium between the intracellular pH (pHi) and extracellular pH (pHex). The composition of the calibration Kıo-clamp buffers were as follows: 20mmol/L NaCl, 110 mmol/L KCl, 1mmol/L CaCl2,1mmol/L MgSO4,18mmol/L D-glucose, and 20mmol/L HEPES that was adjusted to different pH values (6.0-9.0) by titration with NaOH. The pHi calibration was fitted to linear regression, and the fitted parameters were used to generate an equation that converted the ratio channel number (FL-2/FL-3) from the medians of 10,000 cell counts to pHi.

Validation experiments

Control compounds used in this study include Histamine (1 mmol/L) and Omeprazole (1 mmol/L) (both Sigma-Aldrich). Histamine functions as a physiological stimulant while Omeprazole was selected for its prominent properties in hampering stomach acid secretion. Initial experiments were conducted with coffee samples prepared using hot boiling water as treatment to the cells whereby the time course and dose-response acted as the manipulation variables to identify the concentration and treatment time which consequence the most pronounced effect on the IPX (intracellular proton index). Finally, the HGT-1 cells were treated with 2.5 g/mL of each commercial coffee sample (hot-boiled for 10 minutes and let to cool to room temperature).

Statistical Analysis

Minitab version 17 was used in analyzing the HPLC data and flow cytometry data. The chlorogenic acid, caffeine, and NMP content were expressed as mean ± standard deviation. One-way ANOVA analysis with Tukey HSD post hoc test was used to find the significant differences between different groups. A one-sample T-test was performed for the cell viability against 70%. Two sample t-test was used to compare omeprazole or histamine with the non-treated control cells for flow cytometry assay calibration. The Pearson correlation analysis was used to find the correlation between the HPLC quantitative analysis of caffeine, NMP, chlorogenic acid, and its isomers with the gastric release (IPX value). The correlation strength was made based on Cohen (1988), looking at Pearson correlation coefficient value of $0.1 < | r | < .3$ (small correlation), $0.3 < | r | < .5$ (medium/moderate correlation) and $| r | > .5$ (large/strong correlation) (22). The significant differences were set at $p<0.05$.

RESULTS

Quantification of chlorogenic acid and caffeine

Chlorogenic acids and their caffeoylquinic acid isomers, together with caffeine are the main components to be quantified as they are putatively found to cause gastric release. The standard curves were obtained for 5-CQA (Y=7.9415x+65.465, r2 = 0.9999), 3-CQA (Y=4.3181x+36.735, r2= 0.9998), 4-CQA (Y=4.6822x-50.762, r2 =0.9999), and caffeine (Y=5.9455x+153.85, r2= 0.9982). The quantification of chlorogenic acid and caffeine content in different types of instant coffee was tabulated in Table II. Caffeine content was found to be most significantly highest ($p<0.05$) in BC (60,212 ± 212 µg/ml) samples, followed by LS (10,039 ± 4.75 µg/ml), WCA (7260 ± 361 µg/ml), LF (4729 ± 6.48 µg/ml), REG (3289 ± 5.16µg/ml), and WC (2527 ± 1.49 µg/ml). Lastly, the DC samples showed the lowest caffeine compound content, which goes below the LOD value and was indicated as undetected (ND). The total chlorogenic acid (CGA, ug/ml) also showed that BC samples had significantly high ($p<0.05$) chlorogenic acid content while other instant coffee samples showed no significant differences.

Quantification of N-methylpyridinium (NMP) compound N-methylpyridinium is the by-product of degraded alkaloid trigonelline through the decarboxylation process. The identity of NMP peak in every seven different types of the coffee sample tested was confirmed using the validated analytical method and the resulted NMP quantified values were later calculated using the corresponding standard calibration. The results were then tabulated in Table III with the NMP amount depicted as mean ± SD. Among the different coffee samples, it was found that NMP content in black coffee (565 ± 7.59µg/

Table II: The concentration amount (mean ± SD expressed in µg/serving) of the major CQAs and caffeine found in per serving (20g/200ml) of seven different types of instant coffee and the amount of total CGA and caffeine (µg, w/w%) in 2.5 mg/ml of coffee

Instant Coffee	3-CQA ug/serving	4-CQA ug/serving	5-CQA ug/ serving	Total CGA ug/ serving	Total CGA (µg, w/w%)	Caffeine ug/ serving	Caffeine (µg, w/w%)
BC	13,177 ± 199[a]	14,128 ± 170[a]	8474 ± 79[a]	35,779 ± 3027[a]	298 ± 75.70[a]	60,212 ± 212[a]	1503 ± 5.31[a]
DC	767 ± 1.89[c]	1498 ± 2.55[b]	664 ± 12[b]	2929 ± 455[b]	7 ± 8.27[b]	ND	ND
LF	492 ± 5.30[d]	1104 ± 9.27[c]	311 ± 11[e]	1907 ± 416[b]	10 ± 12.11[b]	4729 ± 6.48[d]	118 ± 0.16[d]
LS	1243 ± 15.29[b]	1500 ± 5.86[b]	566 ± 8.31[c]	3309 ± 482[b]	28 ± 12.06[b]	10,039 ± 4.75[b]	251 ± 0.12[b]
REG	191 ± 2.99[e]	931 ± 3.43[c]	19 ± 3.72[f]	1141 ± 484[b]	25 ± 12.81[b]	3289 ± 5.16[e]	82 ± 0.13[e]
WC	78 ± 1.38[e]	643 ± 0.64[d]	63 ± 1.07[f]	784 ± 331[b]	24 ± 11.37[b]	2527 ± 1.49[f]	63 ± 0.04[f]
WCA	1044 ± 2.40[b]	1466 ± 2.98[b]	446 ± 11[d]	2956 ± 513[b]	16 ± 10.39[b]	7260 ± 361[c]	181 ± 9.03[c]

[a,b,c,d,e] denotes significant differences (p<0.05) of total CQA and caffeine between coffee samples
ND – Non-detectable
BC (Black coffee), DC (Decaffeinated coffee,) LF (Low-fat coffee), LS (Low sugar coffee), REG (Regular coffee), WC (White coffee), WCA (White coffee low acid)
CQA – Caffeoylquinic acid
CGA – Chlorogenic acid
SD – Standard deviation
W/W% - Weight percent

ml) was highest (p<0.05) followed by decaffeinated coffee (221 ± 3.68 µg/ml), low sugar (123 ± 20 µg/ml) and regular coffee (95 ± 1.02 µg/ml). Meanwhile, low-fat coffee, white coffee, and white coffee with low acid were found to have the lowest NMP content. The lowest amount of NMP was recorded in instant white coffee (52 ± 0.88µg/ml) although the difference in value was insubstantial in comparison to low-fat coffee (65 ± 0.41µg/ml) and white coffee low acid sample (71 ± 3.24µg/ml).

Secretory activity measurement by flow cytometry

Validation experiment

Before the coffee samples were analyzed through flow cytometry analysis, a set of experiments involving histamine and omeprazole was conducted as a positive control in calibrating the flow cytometry assay (Figure 1). Histamine was used owing to its well-investigated stimulant effect for the secretion of stomach gastric acid (11).

The opposite effect of omeprazole as a potential inhibitor of stomach acid secretion was also utilized in calibrating the flowcytometry (11). Figure 1 shows the intracellular proton concentration of HGT-1 cells when subjected to omeprazole (1 mmol/L) or histamine (1 mmol/L) in comparison with the non-treated HGT-1 cells (p<0.05). From the figure, we could deduce that the HGT-1 cells showed an increase of intracellular proton concentration by 57% compared to the control cells since omeprazole causes inhibition of gastric release. Through a two-sample t-test, the differences between omeprazole treated cells and the non-treated cells were found to be significant in value (T-Value = 17.25 P-Value = 0.000 DF = 3). The findings indicated less gastric release had occurred. As for histamine, the differences with non-treated cells were substantially different (T-Value = -10.53 P-Value = 0.002 DF = 3) with the proton concentration decreases by 21% indicating intracellular proton to be actively secreted. Hence, from this set of experiments, it can be concluded that the assay is applicable in testing gastric release with the coffee samples.

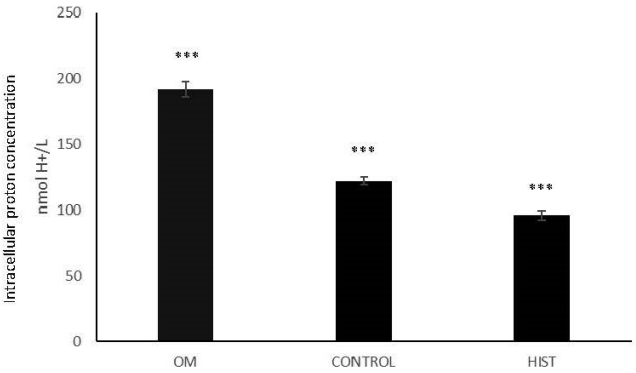

Figure 1: The intracellular proton concentration of HGT-1 cells when treated with omeprazole (OM) (1mmol/L) or histamine (HIST) (1mmol/L) compared to the non-treated control cells (bars shows mean ± SD; two sample t-test: *** = p < 0.05; n = 3-9).

Table III: The amount of N-methylpyridinium (NMP) (µg/serving) shown as means ± SD in each seven different types of instant coffee per serving (20g/200ml) and the amount of NMP (µg, w/w%) in 2.5 mg/ml of coffee.

Instant Coffee	NMP (µg/serving)	NMP (µg, w/w%)
BC	565 ± 7.59[a]	14 ± 0.19[a]
DC	221 ± 3.68[b]	6 ± 0.09[b]
LF	65 ± 0.4[1]e	2 ± 0.01[e]
LS	123 ± 2[0]c	3 ± 0.50[c]
REG	95 ± 1.02[d]	2 ± 0.03[d]
WC	52 ± 0.88[e]	1 ± 0.02[e]
WCA	71 ± 3.24[e]	2 ± 0.08[e]

[a,b,c,d,e] denotes significant differences (p<0.05) of NMP content among the coffee samples
w/w% - weight percent
SD-standard deviation

IPX value indicating secretory activity

The Intracellular Proton Index (IPX) for each coffee was measured when subjected to coffee treatment at a concentration of 2.5mg/ml for 10 minutes. The data presented in Table IV shows the effect of instant coffee on gastric release through the IPX value. A higher IPX value indicates a greater amount of protons remained in the cell, correlating to lower secretory activity and vice versa. The presented data ($p<0.05$) showed that REG (regular) coffee and DC (decaffeinated) coffee have the highest gastric release and were not significantly distinct from each other. The IPX value of proton remained in the cell for both coffee samples was the lowest compared to other coffee samples, indicating high secretory activity.

Meanwhile, coffee samples affecting the lowest release of gastric secretion ($p<0.05$) were LF (low-fat coffee), LS (low sugar coffee), and WCA (white coffee low acid). However, the values obtained by these coffee samples were within approximation to each other, showing insignificant differences in comparison. In addition, the results also demonstrate BC (black coffee) and WC (white coffee) samples had a moderate effect on the gastric release of HGT-1 cells. The IPX value for the samples was neither high nor low, indicating a relatively moderate secretory activity.

The findings of this study were unexpected as it was hypothesized that HGT-1 cells would have the highest gastric release when being subjected to black coffee (BC). The hypothesis was made based on the analytical abundance of selected bioactive compounds such as chlorogenic acid and caffeine in black coffee which are responsible for causing gastric release (Table IV). However, REG (-0.17 ± 0.007) and DC (-0.16 ± 0.005) coffee showed significantly higher gastric secretory activity compared to other coffee samples tested ($p<0.05$). To support the finding, a Pearson correlation was conducted between the HPLC analytical results with flowcytometry proton secretory activity to analyze the possibility of a connection between the amount of caffeine, chlorogenic acid, and NMP in playing an effective role towards the gastric release as indicated through its IPX value.

Table IV: The IPX values (Mean ± SD) of HGT-1 cells when tested with seven types of instant 3-in-1 coffee.

Instant Coffee	IPX (Mean ± SD)
BC	-0.15 ± 0.008[b]
DC	-0.16 ± 0.005[a]
LF	-0.14 ± 0.01[c]
LS	-0.13 ± 0.005[c]
REG	-0.17 ± 0.007[a]
WC	-0.14 ± 0.008[b]
WCA	-0.13 ± 0.008[c]

[a,b,c,d,e] denotes significant differences ($p<0.05$) of IPX value among the coffee samples
IPX- Intracellular proton index

Pearson correlation

A Pearson product-moment correlation was run to verify the relationship between the analytical analysis of selected bioactive compounds to cause gastric release by HPLC analysis and proton secretory activity by flowcytometry analysis. The data obtained for chlorogenic acid, caffeine, and NMP was tabulated in Table V respectively.

For chlorogenic acid compounds, the total chlorogenic acid (TCGA) (mean ± standard deviation) in each coffee sample was correlated with the gastric release (IPX value). Similarly, caffeine (CAFF) and NMP (mean ± standard deviation) were correlated with gastric release represented with the IPX value as well. The R-value or known as the Pearson correlation coefficient indicates the strength of the relationship which follows the cut-off value as suggested by Cohen (1988) (22). As suggested, Pearson correlation coefficient value of $0.1 < | r | < .3$ (small correlation), $0.3 < | r | < .5$ (medium/moderate correlation) and $| r | > .5$ (large/strong correlation) were adopted for this study.

From the tabulated data, it can be deduced that there are strong and positive correlation between TCGA (r = - .609, p = .583) compounds and gastric release (GR) in BC coffee. Similar relationship was also present for CAFF (r = .995, p = .062) whereas the NMP compound in the sample on the other hand exhibits a strong but negative relationship with gastric release (GR) (r = .886, p = .307). Referring to DC coffee sample and its response to gastric release, the result showed medium correlation for TCGA (r = .406, p = .734), strong correlation between CAFF (r = .925, p = .247), and poor negative correlation for NMP (r = -.137, p = .912). The correlation data shows that CAFF (r = .992, p = .080) and NMP (r = .542, p = .635) in LF coffee possess a strong positive correlation with gastric release whereas the TCGA compound was found to be strongly negative (r = -.992, p = .082) in regard to its connection with gastric response. In the analysis of LS coffee, poor positive correlation was found between NMP and gastric release (r = .188, p = .879). However, strong negative connection was found between gastric release and TCGA (r = -.781, p = .403) as well as CAFF (r = -.996, p = .055) for the sample.

The REG coffee correlation data shows that TCGA (r = -.855, p = .347) and NMP (r = -.879, p = .316) have strong and positive correlation with gastric release. As for CAFF (r = -.383, p = .750) in REG coffee, the compound only managed to have medium correlation with gastric release. Next, the correlation data of WC coffee display strong negative correlation between TCGA (r = -.915, p = .264) and NMP (r = -.744, p = .466) in response with gastric release. Meanwhile, medium positive correlation was found between CAFF (r = .304, p = .803) and gastric

release for the coffee sample. Lastly, the correlation data of WCA coffee containing the compounds of TCGA (r = -.894, p = .296) showed strong negative correlation with gastric release and poor positive correlation between the compound CAFF (r = .226, p = .855) and NMP (r = .226, p = .855) were found in response to secretion of gastric acids.

Overall, despite strong correlations were found in most of the compounds for each type of coffee sample and poor to medium correlation in some, the p-value for all of the correlation data is $p > 0.05$ indicating the data is not statistically significant. Non-statistically speaking, the result can be translated into a conclusion that the amount of selected bioactive compounds analyzed by HPLC methodology have no significant correlation with the gastric release through its IPX value. Somehow, the existence of these compounds in instant coffee samples being tested in this study does not exhibit a direct effect on gastric release. Consequently, the results obtained in this study established a similar conclusion with the previous study conducted by Weiss et al., (2010) (11). Other plausible factors within the coffee's complex matrices could be the reason behind the gastric acid stimulation.

DISCUSSION

The selection of coffee beans from different species as well as different technological processes involved in coffee productions would contribute to various amount of bioactive compounds in the final product. The ratios between pure coffee bean being added with other supplementary ingredients such as emulsifier, sodium caseinate, anticaking agent, maltodextrin, and vegetable oil also equally contribute to the final product bioactive compositions. According to The United States of Food and Drug Administration (FDA), most listed ingredients in any food products manufactured are in predominance order with the first order indicates the most dominant amount of content. The majority of the coffee samples tested in this experiment showed the instant coffee powder was listed in the least dominated order which demonstrates the content of other ingredients was much higher than the coffee itself. In addition, the content of bioactive compounds could be further reduced with the existence of added ingredients such as milk or creamer sources which can affect it's analytical recovery (23,24). Even the types of milk being used, either dairy or non-dairy sources used in milk production, would significantly affect the bioactive compounds in coffee (25). Therefore, it is plausible to reserve the preexisting compounds found in coffee as the sole determinant of gastric activities.

Table V: Pearson correlation between gastric release and analytical amount of putative compounds

Coffee	Putative Compounds	R	p-value
BC	TCGA	-0.609	0.583
	CAFF	0.995	0.062
	NMP	0.886	0.307
DC	TCGA	0.406	0.734
	CAFF	0.925	0.247
	NMP	-0.137	0.912
LF	TCGA	-0.992	0.082
	CAFF	0.992	0.080
	NMP	0.542	0.635
LS	TCGA	-0.781	0.403
	CAFF	-0.996	0.055
	NMP	0.188	0.879
REG	TCGA	-0.855	0.347
	CAFF	-0.383	0.750
	NMP	-0.879	0.316
WC	TCGA	-0.915	0.264
	CAFF	0.304	0.803
	NMP	-0.744	0.466
WCA	TCGA	-0.894	0.296
	CAFF	0.226	0.855
	NMP	0.226	0.855

BC (Black coffee), DC (Decaffeinated coffee,) LF (Low-fat coffee), LS (Low sugar coffee), REG (Regular coffee), WC (White coffee), WCA (White coffee low acid)
TCGA – Total chlorogenic acid CAFF – Caffeine NMP- N-methylpyridinium

The effect of additives could either be damaging or even advantageous to the nutrients that exist in a food matrix (26). For instance, protein interactions could mechanistically cause one-third of the chlorogenic acid in a cup of brewed coffee to interact (27). The acidity condition of the coffee matrices further supported this reversible reaction of hydrogen bonding of phenolic compounds and the polar groups of the protein at the hydrophobic pockets of the protein (28) thus limiting the chlorogenic acid capabilities (29). However, in this study, additives may have extended its protective ability towards the polyphenol compounds. Volf and colleagues utilized additives of citric acid, ascorbic acid, sodium chloride, and sodium nitrate which formed protection towards the polyphenols when subjected to high temperature and photo-oxidation (30). Hence, it could be inferred that these additives might protect the interaction of chlorogenic acids with the sweeteners and milk content within the coffee. Few studies have been conducted to confirm the existence of sugar which could lower the amount of bioactive compounds in coffee (31,32). This finding could be one of the contributing factors for the insignificant result of chlorogenic acid between the seven types of coffee being tested as well. Since no data were found regarding the beneficial effect of additives towards caffeine and NMP (N-methylpyridinium), it can be speculated that caffeine and NMP compounds are not included in the protection spectrum of the additives. This could be a reasonable explanation for the current results where a significant difference in the value of caffeine and NMP between the coffee samples was found. Hence, caffeine is susceptible to different processing techniques and any

added ingredients such as milk or creamer which could later hinder its gastric releasing properties.

Consequently, it was expected that these compounds would correlate with gastric release activity. Nonetheless, the p-value for all the correlation data is $p > 0.05$, indicating the data is not statistically significant. This can be translated into saying the amount of the selected bioactive compounds analyzed by HPLC (high-pressure liquid chromatography) methodology have no significant correlation with the gastric release through its IPX (Intracellular Proton Index) value. Somehow, the existence of these compounds in instant coffee has no direct effect on gastric release. Other added ingredients such as sugar and fat could further mask the effect of selected bioactive compounds by disturbing the coffee matrices to have some interactions that reduce the prowess of the selected bioactive compounds in stimulating the gastric release. For instance, the effect of added sugar was published and found to have a significant effect on bioactive contents in tea (31). Other studies had also reached a similar conclusion in indicating sugar's effect on bioactive compounds (32–34). The involvement of the hydroxyl group from sucrose or sugar could mechanistically interact and affect the selected bioactive compounds (35). A study on artificial sweeteners increases the gastrin release which in turn regulates gastric acid secretion activity (36). The influences of sugar on bioactive compounds should be considered as a fundamental determinant in gastrointestinal studies due to its extensive usage in almost all premixed beverages, particularly instant coffee.

Regarding the content of fat in coffee, it is mainly contributed by the creamer or milk sources added into the coffee. A condition known as milk ring or oiling off could trigger instability to the preexisting compounds and affect the chlorogenic acid content in instant coffee (24). Results from these milk sources, the affinity of proteins and polyphenol compounds was discovered to be involved in the reaction of hydrogen bonds (28). The existence of protein interaction would form an interaction with one-third of the chlorogenic acid in a cup of coffee (37). Milk proteins such as whey proteins and casein that exist in dairy products have a high affinity to bind with various bioactive compounds (38), affecting the bioavailability of polyphenols in the human body (39).

In addition, it is also important to point out that the types of creamer mixed into the coffee also play a significant role in gastric release. Generally, the sources of fat in coffees which were identified based on the listed ingredients mainly originated from the dairy sources of cow's milk creamer and skimmed milk whereas the usage of non-dairy sources in WCA and LF coffee, allowed them to be categorized as low-fat. Surprisingly, the WCA and LF coffee samples indicated the coexistence of non-dairy creamer and sugar could reduce the stimulation of gastric release in HGT-1 cells. Research performed had proven that either dairy or non-dairy sources can have a significant effect on the bioactive compounds, possibly affecting the coffee bio-matrices that could hinder the ability to initiate gastric secretion (25). Mechanistic explanation through in vitro/ex vivo showed some binding of bioactive compounds with albumin or casein and 40% of the bioactive compounds being studied were bound to casein. Other 17% of it have persisted throughout the digestive process (27,37). In essence, the biochemical properties of fat content derived from both dairy and non-dairy sources could alter the chemical compositions of coffee thus affecting gastric release as well.

In this study, the decaffeinated coffee (-0.16 ± 0.005) was among the coffee samples which showed the highest response to stimulation of gastric acid release. From the presented data, it was found that the removal of certain caffeine processes could also affect the ability of gastric secretion of HGT-1 cells. The process of decaffeination may produce another unknown compound which has the potential to promote the gastric release. The decaffeinating process involves the removal of caffeine with organic solvents such as dichloromethane, ethyl acetate, or even water/vapour which had gained popularity as an economical alternative compared to the other organic solvent (40). Furthermore, the decaffeination process could also lead to the loss of key flavor components of coffee (41). Hence, the fraction loss of the flavor and aroma needs to be compensated through various devices which could reincorporate the aromatic scent back into the decaffeinated products (40). These processing methods could produce new compounds or interactions that would possibly stimulate gastric release. Limited studies have been conducted on the effect of decaffeinated matrices and the compounds that existed towards gastric release or gastric health. An earlier pivotal study performed by Feldman, Isenberg, & Grossman in 1981 which uses human samples proved that decaffeination has a high potential for promoting gastric release. However, the absence of follow-up study neglected the need of discovering potential compound existed in decaffeinated coffee which could compromise the gut health (42).

Alternatively, ochratoxin A (OTA) contamination could be a plausible explanation behind the gut health issue inflicted by the decaffeinated coffee samples. Ochratoxin A is a prevalent fungal toxin produced by Aspergillus species and Penicillium species (43). The decaffeination process with a non-controlled moisture condition could lead to OTA's presence inside the coffee product (44). In addition, the plausible adulteration of the coffee product and usage of purely instant decaffeinated coffee without any added ingredients (Table I) also increases the risk of OTA contamination. Previous research analysis had disclosed the presence of mycotoxins metabolites

existing inside one hundred coffees that were sold in Spain. Five coffee samples from the total tested samples were positive with Ochratoxin A (OTA) with values that exceeded the permitted levels (45). A study on OTA's effect on gut health provided specific insight on its effect on the high severity of lesion and oocyst indexes at the intestine that damaged the mucosa (46). Moreover, the impact of OTA at the protein level had found significant suppression of TJ proteins which could jeopardize the intestinal integrity (47). This study had identified external factors such as contamination also played a pivotal role in the gastrointestinal properties of coffee mix.

This study has some limitations. The coffee samples used in this current study was purchased from the supermarket which has already been processed and packed. Hence, the instant 3-in-1 coffee composition could not be modified, and the effect of other added ingredients such as food additives on gastric release could not be compared. Moreover, their effects and interactions with the compounds inside the coffee are also unknown. Hence, this study was unable to attain normalization of the wide variety of substances in coffee samples. Instead a certain degree of assumptions has been made using the black coffee as a control sample to observed the gastric release effect of preservatives and other added ingredients in coffee samples. Another area of limitation of the study is the determination of putative compounds that can cause gastric release. In this study, only the most prominent compounds such as caffeine, N-methylpyridinium, chlorogenic acid, and its isomers (CQA-3, CQA-4, CQA-5) were determined. Based on the result in this study, the compounds are not directly correlated to the gastric release effect. Thus, other preexisted compounds inside the coffee should be warranted for further research and analysis, especially in instant 3-in-1 processed coffee. The actual contents of the manufactured products should be investigated beyond the listed ingredients mentioned to allow the study of other compounds constituting the coffee products, rather than simply focusing on the coffee itself.

CONCLUSION

In this study, different types of instant coffee namely black coffee (BC), white coffee low acid (WCA), white coffee (WC), low sugar coffee (LS), low-fat coffee (LF), decaffeinated coffee (DC), and regular (REG) samples were used for quantification of selected bioactive compounds with gastric release properties such as caffeine, N-methylpyridinium, chlorogenic acid and its isomers (CQA-3, CQA4, and CQA-5) using the high-performance liquid chromatography (HPLC). Caffeine (60,212 ± 212 µg/ml) and chlorogenic acid (35,779 ± 3027 µg/ml) content was significantly higher in BC sample. Subsequently, HGT-1 cells were treated with instant coffee samples and analyzed using SNARF-AM dye with flow cytometry analysis machine to determine the gastric release represented by the intracellular proton index (IPX) value. The instant DC coffee samples and REG coffee samples showed a significant gastric release effect with an IPX value of -0.16 ± 0.005 and -0.17 ± 0.007, respectively. Finally, no significant correlation between the quantitative and IPX data was seen. In conclusion, the selected bioactive compounds within the instant coffee samples have no significant effect on gastric release.

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