ABSTRACT

A sensitive, selective, precise and stability-indicating high-performance thin-layer chromatographic (HPTLC) method for analysis of repaglinide both as a bulk drug and in nanoemulsion formulation was developed and validated. The method employed TLC aluminum plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of chloroform/methanol/ammonia/glacial acetic acid (7.5:1.5:0.9:0.1, v/v/v/v). This system was found to give compact spots for repaglinide ($R_f$ value of 0.38 ± 0.02). Repaglinide was subjected to acid and alkali hydrolysis, oxidation, photodegradation and dry heat treatment. Also, the degraded products were well separated from the pure drug. Densitometric analysis of repaglinide was carried out in the absorbance mode at 240 nm. The linear regression data for the calibration plots showed good linear relationship with $r^2 = 0.998 ± 0.032$ in the concentration range of 50-800 ng. The method was validated for precision, accuracy as recovery, robustness and specificity. The limits of detection and quantitation were 0.023 and 0.069 ng per spot, respectively. The drug undergoes degradation under acidic and basic conditions, oxidation and dry heat treatment. All the peaks of the degraded product were resolved from the standard drug with significantly different $R_f$ values. Statistical analysis proves that the method is reproducible and selective for the estimation of the said drug. As the method could effectively separate the drug from its degradation products, it can be employed as a stability-indicating one. Moreover, the proposed HPTLC method was utilized to investigate the degradation kinetics in 1M NaOH.

KEY WORDS: Degradation, high-performance thin-layer chromatography, repaglinide, stability-indicating
testing have been described as per International Conference on Harmonization (ICH) guidelines. The aim of the present work is to develop an accurate, specific, repeatable and stability-indicating HPTLC method for the determination of repaglinide in the presence of its degradation products and related impurities for assessment of purity of bulk drug and stability of its bulk dosage forms. The proposed method was validated as per ICH guidelines.\[11,12\] Acid-induced degradation kinetics were investigated by quantitation of drug by validated stability-indicating HPTLC method.

**Experimental**

**Materials**

Pharmaceutical grade of repaglinide was kindly supplied as a gift sample by Ranbaxy Laboratory, India, used without further purification and certified to contain 99.98% w/w. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, India.

**Instrumentation**

Chromatography was performed in the same manner as reported earlier.\[13\] In brief, samples were spotted in the form of bands of width 6 mm with a Camag microlitre syringe on precoated silica gel aluminum Plate 60 F–254 (20 × 10 cm with 250 μm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai, India) using a Camag Linomat IV (Muttenz, Switzerland). The plates were prewashed by methanol and activated at 60°C for 5 min prior to chromatography. The slit dimension was kept at 5 × 0.45 mm and 10 mm/s scanning speed was employed. The monochromatic bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of chloroform/methanol/ammonia/glacial acetic acid (7.5:1.5:0.9:0.1, v/v/v/v) and 15 mL of mobile phase was used as per chromatography. Linear ascending development was carried out in 20 × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 ± 2°C) at relative humidity of 60 ± 5%. The length of chromatogram run was 8 cm. Subsequent to the development, TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed on Camag TLC scanner III in absorbance mode at 240 nm and was operated by CATS software (V 3.15, Camag). The source of radiation utilized was deuterium and tungsten lamp.

**Calibration curve of repaglinide**

A stock solution of repaglinide (1000 μg mL⁻¹) was prepared in methanol. Standard solutions were prepared by dilution of the stock solution with methanol to give solutions containing repaglinide in a concentration range of 50-800 ng spot⁻¹. Each concentration was spotted thrice on the TLC plate. The data of peak areas and corresponding concentrations were treated by linear least-square regression analysis.

**Method validation**

**Precision**

Precision of the method was determined with the product. An amount of the product powder equivalent to 100% of the label claim of repaglinide was accurately weighed and assayed. System intra-day repeatability was determined by six replicate application and six times measurement of a sample solution at the analytical concentration of 300 ng spot⁻¹. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of percentage relative standard deviation (% RSD) and standard error (S.E.), and were found to be less than 2%. Intermediate precision was assessed by the assay of two sets of six samples on different days (inter-day precision). The intra- and inter-day variation for determination of repaglinide was carried out at three different concentration levels: 100, 300 and 500 ng spot⁻¹, respectively.
Accuracy (as recovery)

The analyzed samples were spiked with extra 50%, 100% and 150% of the standard repaglinide, and the mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was done to check for the recovery of the drug at different levels.

Robustness

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions like chloroform/methanol/ammonia/glacial acetic acid (7.7:1.3:0.9), (7.3:1.7:0.9), (7.5:1.7:0.5), (7.5:1.3:1.1), (7.7:1.5:0.7) and (7.3:1.5:1.1) were tried (keeping volume of glacial acetic acid constant) and chromatograms were run. The amount of mobile phase, temperature and relative humidity was varied in the range of ± 5%. The plates were prewashed by methanol and activated at 60 ± 5°C for 2, 5 and 7 min prior to chromatography. Time from spotting to chromatography and from chromatography to scanning varied from 0, 20, 40 and 60 min. Robustness of the method was done at three different concentration levels: 100, 300 and 500 ng spot⁻¹.

Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for repaglinide in the sample was confirmed by comparing the Rf and spectra of the spot with that of standard. The peak purity of repaglinide was assessed by comparing the spectra at three different levels; i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot.

Sensitivity

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ were determined by standard deviation method. For the determination of LOD and LOQ, blank sample was injected in triplicate and peak area of this blank was recorded. The LOD and LOQ were calculated using the slope (S) of the calibration curve and standard deviation (α) of the blank sample by the following formulae: LOD = 3α/S and LOQ = 10α/S, where α is the standard deviation of the blank response and S is the slope of the calibration curve.

Nanoemulsion analysis for repaglinide

To determine the content of repaglinide in nanoemulsion formulation. An accurately weighed quantity of formulation equivalent to 20 mg RPG was extracted with 100 mL methanol by sonication for 30 min and volume was made up to 100 mL. The resulting solution was centrifuged at 5000 rpm for 5 min and supernatant was analyzed for drug content. Two μL (400 ng spot⁻¹) of the filtered solution was applied to a TLC plate followed by development and scanning as described earlier. The analysis was repeated in triplicate. The possibility of excipient interference in the analysis was studied.

Forced degradation of repaglinide

Acid and base-induced degradation

Fifty mg of repaglinide was dissolved in 25 mL of methanolic solution of 1M HCl and 1M NaOH. These mixtures were refluxed for 6 h at 90°C in the dark in order to exclude the possible degradative effect of light. The resultant solutions were diluted 10 times, and applied on the TLC plate in triplicate (2 μL, i.e., 400 ng spot⁻¹). The chromatograms were run as described above.

Photo-UV degradation

Fifty mg of repaglinide was dissolved in 25 mL of methanol and exposed to direct sunlight for 3 days and UV irradiation for 10 days in UV stability chamber. The resultant solutions were diluted 10 times, and applied on the TLC plate in triplicate (2 μL, i.e., 400 ng spot⁻¹) and then chromatograms were run as described above. In all degradation studies, the average peak area of repaglinide after application (400 ng spot⁻¹) of three replicates was obtained.

Dry and wet heat degradation

The standard drug was placed in an oven at 80°C for 2 h to study dry heat degradation and the stock solution was refluxed for 2.0 h on boiling water bath for wet heat degradation.

Peroxide-induced degradation

To 25 mL of methanolic solutions of repaglinide, 25 mL of hydrogen peroxide (30.0%, v/v) was added. The solutions were heated at temperature 65°C in boiling water bath for 6 h to remove completely the excess of hydrogen peroxide. The resultant solutions were diluted appropriately and applied on TLC plate (400 ng spot⁻¹) and then chromatograms were run as described above. In all degradation studies, the average peak area of repaglinide after application (400 ng spot⁻¹) of three replicates was obtained.

Detection of related impurities

The related impurities were determined by spotting higher concentrations of the drug so as to detect and quantify them. Repaglinide (2000 mg) was dissolved in 100 mL of methanol, and this solution was termed as sample solution (20 mg/mL). Two mL of the sample solution was diluted to 100 mL with methanol
and this solution was termed as standard solution (0.4 mg/mL). One microliter of both the standard (400 ng spot<sup>−1</sup>) and the sample solution (20,000 ng spot<sup>−1</sup>) were applied on TLC plate and the chromatograms were run as described above.

Study of base-induced degradation kinetic

Accurately weighed 100 mg of the drug was dissolved in 100 mL methanol. Twenty mL of this standard solution was transferred into 100 mL double neck round bottom flask. To it 20 mL of 1M NaOH was added and refluxed at different temperatures (50, 70 and 90°C). At specified time intervals the contents of the flask were quantitatively transferred to 10 mL volumetric flasks and estimated by HPTLC method. The experiment was carried out in triplicate. The concentration of the remaining drug was calculated for each temperature and time interval. Data was further processed and degradation kinetic constants were calculated.

Results and Discussion

Mobile phase optimization

The TLC procedure was optimized with a view to developing a stability-indicating assay method. Both the pure drug and the degraded drug solution were spotted on the TLC plates and run in different solvent systems. Initially, chloroform/methanol/ammonia was tried. The mobile phase chloroform/methanol/ammonia (7.5:1.5:0.9, v/v/v) gave good resolution with R<sub>f</sub> value of 0.38 for repaglinide but typical peak nature was missing. Also, the spot for repaglinide was slightly diffused. Addition of 1.5 mL of glacial acetic acid to the above mobile phase improved the spot characteristics. Finally, the mobile phase consisting of chloroform/methanol/ammonia/glacial acetic acid (7.5:1.5:0.9:0.1, v/v/v/v) gave a sharp and symmetrical peak with R<sub>f</sub> value 0.38 ± 0.02 [Figure 1]. Well-defined spots were obtained when the chamber was saturated with the mobile phase for 30 min at room temperature.

Validation

Linearity

The linear regression data for the calibration curves (n = 3) showed a good linear relationship over a concentration range of 50-800 ng spot<sup>−1</sup> with respect to the peak area. The regression equation was found to be Y = 12.138 + 74.235 with correlation coefficient (r ± S.D.) of 0.998 ± 0.032. No significant difference was observed in the slopes of standard curves (ANOVA, P > 0.05).

Table 1: Precision (n = 3)

| Amount (ng spot<sup>−1</sup>) | Intra-day precision | Inter-day precision |
|-------------------------------|---------------------|---------------------|
|                               | Mean area | SD    | % RSD | SE  | Mean area | SD    | % RSD | SE  |
| 100                           | 4738.65   | 13.55 | 0.2859| 7.823| 4625.43   | 12.43 | 0.3539| 7.1766|
| 300                           | 15342.95  | 25.30 | 0.1648| 14.6073| 14832.75  | 14.57 | 0.2098| 8.4122|
| 500                           | 25683.54  | 55.63 | 0.2169| 32.1189| 26893.54  | 45.44 | 0.1285| 26.2355|

RSD: Relative standard deviation

Precision

Precision was considered at two levels of ICH suggestions i.e., repeatability and intermediate precision. Repeatability of sample application was determined as intra-day variation whereas intermediate precision was determined by carrying out inter-day variation at three different concentration levels, 100, 300 and 500 ng spot<sup>−1</sup> in triplicates; % relative standard deviation (RSD) was found in the range between 0.1648-0.2859% and 0.1285-0.3539%, respectively [Table 1]. The low value of % RSD (<1%) reveals an excellent precision of the method.

Accuracy (as recovery)

The proposed method when used for extraction and subsequent estimation of repaglinide from bulk drug and nanoemulsion formulation formed after spiking with 50%, 100% and 150% of additional drug afforded recovery of 98.79-99.61%, as listed in Table 2.

Robustness

The SD of peak areas was calculated for each parameter and RSD was found to be less than 2%. The low values of %RSD as shown in Table 3 indicated robustness of the method.

Specificity

The peak purity of repaglinide was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot, i.e., r<sup>2</sup> (S, M) = 0.9997 and r<sup>2</sup> (M, E) = 0.9979. Good correlation (r<sup>2</sup> = 0.9998) was also obtained between standard and sample spectra of repaglinide.

Sensitivity

The signal-to-noise ratios 3:1 and 10:1 were considered as LOD and LOQ, respectively. The LOD and LOQ were found to be 0.023 and 0.069 ng spot<sup>−1</sup>, respectively.

Nanoemulsion analysis for repaglinide

A single spot at R<sub>f</sub> 0.38 was observed in the chromatogram of the drug samples extracted from prepared formulation. There was no interference from the excipients commonly present in the formulation. The drug content was found to be 99.39 ± 0.35% with a %RSD of 0.62 for six replicate determinations. It may therefore be inferred that degradation of repaglinide had not occurred in the prepared nanoemulsion formulations that were analyzed by this method. The good performance of the method
indicated the suitability of this method for routine analysis of repaglinide in pharmaceutical dosage form.

**Forced degradation of repaglinide**

**Acid and base-induced degradation**

The chromatogram of the acid-degraded sample for repaglinide showed peak at R$_f$ 0.19 and 0.43 [Figure 2a]. The chromatogram of the base-degraded sample showed peak at R$_f$ value of 0.20, 0.25, 0.29 and 0.51 [Figure 2b]. The areas of the degraded peaks were found to be less than the area of standard drug concentration (400 ng spot$^{-1}$) indicating that repaglinide undergoes degradation under acidic and basic conditions.

**Photo-UV degradation**

The photo-degraded sample showed one additional peak at R$_f$ 0.22 when drug solution was left in daylight for 3 days. The drug was degraded when exposed to UV irradiation for 10 days and showed additional peaks at R$_f$ value of 0.53.

**Dry and wet heat degradation**

The samples degraded under dry heat and wet heat conditions [Figure 3a and b] showed additional peaks at R$_f$ 0.16, 0.46 and 0.51, respectively. The spots of degraded products were well resolved from the drug spot.

**Peroxide-induced degradation**

The sample degraded with 30% v/v hydrogen peroxide showed additional peaks at R$_f$ value of 0.41, 0.46 and 0.62 [Figure 4a]. The spots of degraded products were well resolved from the drug spot.

| Table 2: Accuracy, as recovery studies (n=6) |
|---|---|---|---|---|---|
| Excess drug added to analyte (%) | Theoretical content (ng) | Amount obtained (ng) | (%) | Recovery | % RSD | SE |
| 0 | 200 | 198.76 | 99.38 | 0.486 | 0.572 |
| 50 | 300 | 298.84 | 99.61 | 0.354 | 0.584 |
| 100 | 400 | 396.61 | 99.15 | 0.515 | 1.153 |
| 150 | 500 | 493.97 | 98.79 | 0.286 | 0.654 |

RSD: Relative standard deviation

| Table 3: Robustness testing (n=6) |
|---|---|---|---|
| Parameters | SD | % RSD | SE |
| Mobile phase composition (±0.2 mL) | 1.36 | 1.14 | 0.785 |
| Amount of mobile phase (±5%) | 1.08 | 0.85 | 0.623 |
| Temperature (±5%) | 1.34 | 1.04 | 0.773 |
| Plate pretreatment (±5 min) | 0.89 | 0.67 | 0.513 |
| Relative humidity (±5%) | 1.44 | 1.21 | 0.831 |
| Time from spotting to chromatography (±20 min) | 0.75 | 0.63 | 0.433 |
| Time from chromatography to scanning (±20 min) | 0.93 | 0.74 | 0.536 |

RSD: Relative standard deviation

**Figure 2:** Forced degradation study; (a) Chromatogram of acid-treated RPG (peak 1: Degraded, Rf: 0.19; peak 2: Repaglinide, Rf: 0.38; peak 3: Degraded, Rf: 0.43); (b) Chromatogram of base-treated RPG (peak 1: Degraded, Rf: 0.20; peak 2: Degraded, Rf: 0.25; peak 3: Degraded, Rf: 0.29; peak 4: Repaglinide, Rf: 0.38; peak 5: Degraded, Rf: 0.51)

**Figure 3:** Chromatograms of (a) dry heat-degraded RPG (peak 1: Degraded, Rf: 0.16, peak 2: Repaglinide, Rf: 0.38, peak 3: Degraded, Rf: 0.45); (b) wet heat-degraded RPG (peak 1: Repaglinide, Rf: 0.38, peak 2: Degraded, Rf: 0.51)
Detection of the related impurities

The spots other than the principal spot (repaglinide) from the sample solution were not as intense as the principal spot from the standard solution. The sample solution showed one additional spot at \( R_f = 0.06 \) [Figure 4b]. However, the peak area of the additional spot was found to be much less as compared to the peak area of the principal spot from the standard solution.

Degradation kinetics

In basic medium, a decrease in the concentration of the drug with increasing time was observed. At the selected temperatures (50, 70 and 90°C), the degradation process followed pseudo first-order kinetics. Apparent first-order degradation rate constant and half-life were obtained from the slopes of the straight lines at each temperature. Data obtained from first-order kinetics treatment was further subjected to fitting in Arrhenius equation \( K = A e^{-E_a/RT} \), where \( K \) is rate constant, \( A \) is frequency factor, \( E_a \) is energy of activation (Cal mol\(^{-1}\)), \( R \) is gas constant (1.987 cal deg\(^{-1}\) mol\(^{-1}\)) and \( T \) is absolute temperature (°K). A plot of \( (2 + \log K_{obs}) \) values versus \( (1/T \times 10^3) \) the Arrhenius plot was obtained [Figure 5], which was found to be linear in the temperature range of 40-90°C. The degradation rate constant at room temperature (K25°) is obtained by extrapolating the resulting line in the Arrhenius plot to 25°C [Figure 6] and was found to be \( 12.4 \times 10^{-2} \) h\(^{-1}\) and calculated \( t_{1/2} \) and \( t_{0.9} \) are 5.59 and 0.848 h respectively.

Conclusion

The HPTLC technique developed for RPG was found specific, accurate and stability-indicating. Statistical analysis proves that the method is repeatable and selective for the analysis of repaglinide as bulk drug and in nanoemulsion formulations without any interference from the excipients. The method can be used to determine the purity of the drug available from various sources by detecting the related impurities. The above results showed the suitability of the proposed method for basic-induced degradation kinetic study of repaglinide. The degradation rate constant, half-life and shelf life of repaglinide can be predicted. As the method separates the drug from its degradation products, it can be employed as a stability-indicating one.

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Development and evaluation of fixed dose bi-therapy sublingual tablets for treatment stress hypertension and anxiety

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ABSTRACT

Objective: A stress induced rise in the blood pressure. Some believe that patients with hypertension are characterized by a generalized state of increased anxiety. Aim: The purpose of this study is to prepare a fixed dose bi-therapy using bisoprolol hemifumarate (BH) as antihypertensive drug and buspirone hydrochloride (BuHCl) as anxiolytic drug, which can be used to treat both diseases concomitantly. Using sublingual tablets is hopeful to improve the BuHCl poor oral bioavailability and to facilitate administration to patients experiencing problems with swallowing. Materials and Methods: A total of 5mg BH and 10mg BuHCl were selected based on compatibility study. A 3×22 full factorial design was adopted for the optimization of the tablets prepared by direct compression method. The effects of the filler type, the binder molecular weight, and the binder type were studied. The prepared formulae were evaluated according to their physical characters as hardness, friability, disintegration time (new modified method and in vivo disintegration time) and wetting properties. In vitro drugs dissolute, permeation through the buccal mucosa and the effect of storage were analyzed by a new valid high pressure liquid chromatography (HPLC) method. Bioavailability study of the selected formula study was carried out and followed by the clinical. Results: The optimized tablet formulation showed accepted average weight, hardness, wetting time, friability, content uniformity, disintegration time (less than 3 min). Maximum drug release could be achieved with in 10 min. In addition enhancing drug permeation through the buccal mucosa and, the maximum concentration of the drug that reached the blood was in the first 10 min which means a rapid onset of action and improved the extent of both drug’s absorption. Conclusion: The results revealed that sublingual (F6) tablets containing both drugs would maintain rapid onset of action, and increase bioavailability. BuHCl with BH can be attributed to the marked decline in DBP and SBP. That led to a reduction in the MAP.

KEY WORDS: Bisoprolol hemifumarate, buspirone hydrochloride, sublingual tablets; transmucosal study, bioavailability study

Stress of modern life and work can be the cause of many diseases, for example; anxiety, depression, hypertension, and an increased risk of cancer and coronary heart disease.

Some believe that patients with the hypertension are characterized by a generalized state of increased anxiety, which has been reported in those with a family history of the high blood pressure (BP).[1,2]

Hence, the need for combination therapy of anxiolytic drug and antihypertensive drug is common, especially for the hypertensive patients who suffering from daytime alertness.

FDC had many advantages as convenience, cost savings and an increase the patient compliance.[3] This study is concerned in the formulation and evaluation of bisoprolol hemifumarate (BH) and buspirone HCl (Bu) combination therapy in sublingual tablets as a combination of Beta blocker (BH) and antianxiety (Bu) which both induced a significant decrease in BP. They reduced the mean arterial pressure (MAP) through decreasing the BP and reducing
the cardiovascular responses to the 5-HT1A receptor agonists.\textsuperscript{[4]}

It was useful for long-term treatment of anxiety; another alternative is the use of adrenergic beta-blocking drugs.\textsuperscript{[13]} Furthermore, by investigating the antihypertensive effect of 5-HT1A agonist, it was found that the chronic administration of Bu, produced a significant reduction in BP\textsuperscript{[6]} The use of certain beta-adrenoceptor antagonists, such as propranolol and metoprolol, might improve the antiepileptic potential of benzodiazepines.\textsuperscript{[7]} In addition, Beta-adrenoceptor blockers may be useful as adjunctive medication in the treatment of depression. As they show affinities to 5-HT1A.\textsuperscript{[8]}

BH is a selective beta-1 receptor blocker and is an alternative first-line choice in the treatment of heart failure and congestive heart failure. It almost completely absorbed from the gastrointestinal tract with small first-pass effect (<10 %) in man, the plasma elimination half-life is 10-12 hours, resulting in the duration of action of 24 hours. The high absorption rate and small first-pass effect result in an absolute bioavailability of 88%. Concomitant food intake does not delay the absorption.\textsuperscript{[9]} The molecular weight of bisoprolol is 325.443 g/mol.

BH leads to 46% reduction in sudden death after 1 year. BH administered once daily appears to be an effective and safe as an antianimal agent. It acts essentially through the reduction of myocardial oxygen consumption.\textsuperscript{[10]}

Bu is structurally unrelated to the benzodiazepines but possesses comparable anxiolytic activity, especially in patients in whom daytime alertness is particularly important.\textsuperscript{[11]} Because Bu lacks hypnotic, anticonvulsant and muscle relaxant properties, it has been termed “anxioselective”. It seems unlikely to generate the problems of dependence, abuse and rebound. Bu appears to be efficacious and well tolerated in the treatment of generalized anxiety disorder in elderly patients.\textsuperscript{[12]} It rapidly absorbed from the gastrointestinal tract reaching peak plasma concentration within 40 to 90 minutes after an oral dose. The systemic bioavailability is low and extensive first-pass effect (>90 %). Concomitant food intake delays the absorption. Half-life of Bu ranged from 2h to 3h. The molecular weight of buspiron is 385.50314 g/mol.\textsuperscript{[9]}

The sublingual route for drug administration has been associated with numerous advantages over oral administration as avoidance of both hepatic and intra-alimentary canal metabolism; convenient taking and high bioavailability furthermore, the sublingual route of administration is useful when an immediate response to drug action is desired furthermore, the inconvenience associated with the administering standard dosage forms applies to people who are ill in bed and to those actively working patients who are busy or traveling and who have no access to water.\textsuperscript{[13]}

In this context, a sublingual tablet containing bi therapy of BH and Bu was developed and evaluated for various pharmaceutics properties including dissolution and stability testing to be used as a promising dosage form easy for handling of elderly patients suffering from stress hypertension and anxiety.

Materials and Methods

Materials

BH was purchased from Merck (Barcelona, Spain), and BuHCl was purchased from Bristol Mayer Squibb (New York, USA). Avicel PH 101, Avicel PH 102, polyethylene glycol 4000, polyethylene glycol 6000, Ac-Di-Sol, magnesium stearate and aspartame purchased from Fluka; Germany. Mannitol (El –Nasr Pharmaceuticals, Egypt). Anhydrous lactose, polyvinyl pyrrolidone K30 and polyvinyl pyrrolidone K90 (Sigma; USA).

Methods

Drug-drug and drug – excepients interaction

Differential scanning calorimetric (DSC) study (Differential Scanning Calorimeter, Shimadzu DSC-50. Japan) and Infrared spectroscopy (IR) (IR spectrometer, Shimadzu IR-470. Japan) were used to characterize (BH), (Bu), mixtures of both BH and Bu (M), and the mixture of (M) with each excipients (1:1 w/w).\textsuperscript{[14]}

Factorial design for tablet formulation

3×2\textsuperscript{2} factorial design was used for tablet preparation. Two factors were used at two levels as follows; (1) Binders (low particle size and high particle size). (2) Diluents (mannotol and lactose) in order to formulate 4 formulas.

The design was repeated 3 times for each binder as; Avicel (PH 101 and PH 102), PEG (PEG 4000 and PEG 6000) and PVP (K30 and K90) in order to formulate twelve formule.

Tablet preparation

The tablet formulae were directly compressed after sieving using single punch tablet machine with 7 mm flat-faced punch [The composition as presented in table 1]\textsuperscript{[15]} (Single punch tablet machine, Shang Hai Hua’s Mao industrial and commercial Co .China). The compaction force was maintained during compression at 20 KN to maintain the tablets porosity. Ingredients of each formula were mixed using the geometric dilution method. The produced mixture passed through a 200 μm sieve and retained on a 100μm sieve in order to maintain the particle size of all components within the range of 75–106 μm. The produced mixture was finally mixed with magnesium stearate for 2 minutes.

Physical evaluation

Tablets’ hardness and tablets’ friability were measured according to the United States pharmacopeia\textsuperscript{[16]} using a tablet hardness tester (Hardness Tester, D.R. Schleunger, 6D tablet tester, Germany) and a friability tester (Friabilator, Van Ceuard, PNC, Germany) respectively. The average hardness and the friability percent were calculated.
Table 1: The composition of different BH and BuHCl mixture sublingual tablets

| Formula NO.* | 68% Diluent | 10% Binder |
|--------------|-------------|------------|
| 1            | Mannitol    | PVP K30    |
| 2            | Mannitol    | PEG 4000   |
| 3            | Avicel PH 101 | PEG 6000 |
| 4            | PVP K 90    |            |
| 5            | PEG 6000    |            |
| 6            | Avicel PH 102 |        |
| 7            | Lactose     | PVP K30    |
| 8            |            | PEG 4000   |
| 9            | Avicel PH 101 |        |
| 10           | PVP K 90    |            |
| 11           | PEG 6000    |            |
| 12           | Avicel PH 102 |        |

*All formulae contain: Bisoprolol hemifumarae (5%w/w) and buspirone hydrochloride (10% w/w) Sweeting agent 1% Aspartame. Disintegrant 5% Ac Di Sol. Lubricant 1% Mg Stearate. BH: Bisoprolol hemifumarate, BuHCl:buspirone hydrochloride, PEG: polyethylene glycol, PVP: poly vinyl pyrrolidone

**Tablets wetting time (WT) and tablets wetting absorption ratio (WAR)**

Tablets WT was measured by a modified procedure from that reported by Bi et al. 1999.[16] The procedure was modified as follows: Five circular tissue paper of 10 cm diameter were placed in a petri dish containing an eosin dye solution in water (10 ml of 0.05% w/v). A tablet was placed carefully on the surface of the tissue paper. The time required for the dye solution to appear on the upper surface of tablet. A WT was recorded using a stopwatch. The water absorption ratio was calculated using the following equation: \[ \text{WAR} = \frac{(W_b - W_a)}{W_a} \] The test was repeated three times.

Where \( W_a \) and \( W_b \) are the weights before and after water absorption, respectively.[17]

**Disintegration time (DT)**

**Measurement of DT by a modified apparatus**

Instead of the conventional disintegration apparatus described in United States Pharmacopoeia USP, a modified Shukla et al., 2009[18] method was used, 500 ml of phosphate buffer pH 6.8 maintained at 37 ± 0.5°C and stirred with a paddle at 50 rpm. The tablets were placed in a sinker. (Dimensions of the sinker: 5 cm in height and 3.5 cm in width, mesh #10).

**In-vivo oral disintegration time**

A test was performed on six healthy adult volunteers. Before the test, the volunteers were asked to rinse their mouth with distilled water. The tablet was placed under the subject’s tongue, and immediately a chronometer was started until complete disintegration. The chronometer was stopped, and the time recorded.[19] Permission to carry out this work was obtained from the Institutional Ethics Committee (National Organization of Drug Control and Research [NODCAR], Giza, Egypt).

**HPLC method for the determination of (BH) in the presence of (Bu)**

The stock solutions of BH and Bu were prepared in a phosphate buffer pH 6.8 at a free base concentration of 1mg/ml (A) respectively. Secondary standard solution for BH and Bu mixture (M) 10μg/ml was prepared as a stock solution. Working standard solutions of (M), 1, 2, 5 μg/ml, 500, 200, 100, 50, 20, 10 ng/ml were prepared from secondary standard solution by dilution with phosphate buffer pH 6.8 respectively.

HPLC procedure was proposed using a 30mm × 4mm (Luna eclips® XBB C18 analytical column) with the aid of a guard column. The mobile phase consisted of acetonitrile and 0.01M phosphate buffer (pH 5.5) at a ratio (50:70) v/v. The absorbance of the prepared solutions was measured at λ max 229 nm. Triplicate injections were made for each sample.

The assay method was done and validated with respect to intra- and inter-day accuracy and precision as per ICH guidelines for 3 days.

**In vitro dissolution study**

Tablet formulas were introduced individually into each vessel containing 500 ml of phosphate buffer solution pH 6.8, 37 ± 0.5°C and at a speed of 50 rpm. (USP Apparatus ΙΙ) (Dissolution tester, Pharma test, PTZ, Germany). Aliquots of 5 ml were withdrawn from the dissolution medium at 5, 10, 15, 20, 30, 45 and 60 minutes. The drug content was determined by HPLC method. Each experiment was carried out in triplicate. The test was adopted after several trial on the modified procedures adopted by Aburahma et al., for evaluation of sublingual tablets containing vinpocetine.[20]

**In vitro permeation through the buccal mucosa**

A test performed using the Franz-type diffusion cell. The buccal mucoa was obtained from a local slaughterhouse (goat buccal mucosa) and used within 3 hours of slaughter. The tissue was stored in a phosphate buffer at 4°C upon collection.

The medium was equilibrated at 37°C ± 0.5°C and stirred 25rpm over a 3-hr. An amount of the drug released from these formulas was determined by the HPLC method.

The percent drug permeated to time was constructed for each experiment, and the slopes of these graphs were used to calculate the permeability coefficients (PC: cm / min) as follows:

\[ PC = \text{slope} \times \frac{V_d}{S} \]

Where \( V_d \) is the volume of the donor solution.

S: is the surface area of the tissue (1.77 cm²).[21]
Accelerated stability testing

The selected formulae were stored at 60°C and 75% relative humidity (maintained using a saturated solution of NaCl) for 3 weeks.[22] The stored tablets were examined visually for any changes in color and/or appearance every week. Physical evaluation of the tablets, including dissolution was repeated at the end of the storage period as the same procedures adopted for the fresh tablets.

Dissolution profiles of fresh and stored tablets were compared according to the model independent mathematical approach of Moore and Flanner, 1996.[23] The similarity factor (f2) was calculated according to the following equation:

\[ f_2 = 50 \log \left\{ 1 + \left( \frac{1}{n} \sum_{t=1}^{n} (R_t - T_t)^2 \right)^{1/2} \right\} \]

Where \( n \) is the number of sampling points. \( R_t \) and \( T_t \) are the mean percent dissolved of the reference (fresh) and the test (stored) at time \( t \) respectively. \( f_2 \) represents a logarithmic transformation of the sum of squared error of differences between the reference and test products over all time points.

Bioavailability of BH and Bu in the selected formulae

A simple crossover design was applied on three phases, using male Albino rabbits (Albino rabbits weighing 2.0-3.0 kg. They were obtained from veterinary service (NODCAR) Egypt.). They were randomly divided into three groups, each containing six rabbits. A group received an oral dose of the market products (Concor® and Buspar®), in a dose 1 mg/Kg/day[24] and 2 mg/Kg/day[25] of the body weight in each phase respectively. The other two groups received F6 and F12 sublingually [The composition was as in table 1] respectively after suitable washout period. The optimizing buccal tablet formula were inserted sublingually and positioned in such a way that, the tablet surfaces came into contact with the ventral tongue and the floor of the mouth of anesthetized rabbits for 3 minutes after shifting the tongue. The rabbits were anesthetized with pentobarbital (25 mg/kg).[26]

All animals were handled in agreement with the ethical principles in animal experimentation adopted by the Ethics Committees Accreditation of Laboratory Animal Experimentation Care (AAALAC) with protocol no. 25/2002.

A volume of blood samples (2.0 ml) was drawn from the terminal veins of the ears at the following time points: 0 (prior to dosing), 5, 10, 15, 20, 30, 60, 90, 120 and 240 minutes and collected in heparinized tubes. Plasma samples were separated and were deproteinized by acetonitrile then analyzed using a HPLC (Agilent 1100). A modified Braza et al., 2002,[27] and a modified Pehourcq,[28] were used and validated for the determination of BH and Bu respectively. Triplicate injections were made for each sample.

The pharmacokinetic parameters were calculated and statistically compared; Cmax(μg/ml), Tmax(Hours), Auc (0-4) (μg.h/ml), Auc (0-∞) (μg.h/ml) and Relative Bioavailability.

The pharmacokinetic data were computed using Kinetica 2000 Version 3.0 (Inna Phase Corporation, USA).

Clinical study

Thirty patients with primary hypertension (age: 50.9 ± 2.38 years, weight: 83.9 ± 2.81 kg, BP: 165.8 ± 3.90/102.5 ± 1.86 mm Hg, heart rate: 63.6 ± 2.98 min−1) were included in this study. The study protocol was approved by the Human Ethics Committee of NODCAR. The procedures followed in this study were in accordance with institutional guidelines. Written informed consent was obtained from all the enrolled patients. Patient compliance was documented on the patient’s case report form. Safety was assessed throughout the study and included monitoring of adverse events, concomitant medications, routine laboratory safety tests (creatinine clearance, Hematology, serum chemistry, and urinalysis values), and physical examination findings.

The test was performed to compare the pharmacodynamic effect of sublingual tablet (F6) to commercial oral tablet (5mg Concor® and 10mg Buspar®) by studying the change of diastolic blood pressure (DBP) and systolic blood pressure (SBP) and recorded them. The sublingual tablet (F6) and commercial oral tablet (5mg Concor® and 10mg Buspar®) were given once daily. The non-blind, three treatments, three periods and randomized crossover study were followed. Under this design, a third of the subjects were given orally 5 mg Concor®, the other third was given orally 10 mg Buspar® and the last group were given F6 buccally (The composition in table 1). BP was measured for 6 hours. The response to the formula was expressed by the area under the curve of the mean arterial pressure (MAP-AUC). The difference of the BP peak induced by both drugs expressed as the change in both and MAP The data collections at different time intervals as 10, 20, 30, 60, 90, 120, 180, 240 and 360 minutes recorded with Mercurial sphygmomanometer. The MAP was calculated according to the following equation followed by statistical analysis.

\[ \text{MAP} = \text{DBP} + \frac{1}{3}(\text{SBP} - \text{DBP}) \]

Statistical analysis

All tests were conducted in triplicates. The results were expressed as the mean ± SD followed by paired t test. One-way Analysis of Variance (ANOVA) was applied to assess the significance of the effect of storage on the physical properties of the tested formulae and the fresh formulae (In All experiments). Two-way ANOVA was applied to assess the significance of the effect of formulation and subject factors on the pharmacokinetic parameters, and the pharmacodynamic parameters of the tested formulae and the oral commercial formulae Concor® and Buspar® tablets. Tukey’s HSD (Honestly Significant Difference) test and Guassian’s test for multiple
comparisons were then performed to determine the source of difference respectively using SPSS® software version 7.5 (SPSS Inc., Chicago, IL). Differences are considered to be significant at $P < 0.05$.

**Result and Discussion**

**Drug-drug and drug – excipient’s interaction**

DSC thermo grams and IR spectrum of M and M-excipient mixtures revealed that; there was not any significant change in the onset (thaw point), endothermic peak or the characteristic bands of each drug as in Figure 1. The insignificant change in the endothermic peak (DSC) or the characteristic bands of each drug (IR) revealed to the absence of possible chemical or physical interaction between both drugs

**Physical characterization**

**Hardness**

All formula showed hardness values ranging from 2 to 5, which are within the pharmacopoeia acceptable ranges of 2-8Kg (USP, 2009) except F8 [Table 2]. Taking the binder factor in consideration, significant difference occurred when changing the binder of smaller particle size with that of larger particle size. The value of hardness is increased ($P$-value=0.158, $SE=0.156$). The analysis of interaction revealed that; mannitol had the lowest hardness value with low particle size binder ($P$-value=0.138, $SE=0.062$) (Data not shown).

The hardness results suggest that the particle size influences of the binder use were more than the type of binder used ($p < 0.05$) and that agreement with Bolourchian *et al.,* 2009.

**Friability**

All tablets accepted friability percent, i.e., less than 1%. All the factors studied had a significant effect on the tablets’ friability ($p < 0.05$). Studying the interaction found that Diluents*PEG interaction only had a significant effect on the tablets’ friability ($p > 0.05$) ($P$-value=0.369, $SE=0.100$) (Data not shown). All tablets have accepted friability because it was reported that the binder concentration at 10% resulted in a decrease in the fracture of the tablets but caused compression problems like stickiness to the punches of compression machine. However, the addition of 1% (w/w) magnesium stearate as a lubricant improves tablets’ compression and reduces its friction with compression machine and has the least effect on the tablet characteristics such as the disintegration time, the hardness and the friability of the tablets.

**Tablets WT and WAR**

Tablets prepared to use a different formula showed different WT and WAR as in Table 2. A ranking of the waiting time and the wetting ratio indicated that F 7 has the tallest WT and the lowest wetting ratio due to its highest hardness value and vice versa in F6 and F12. The reduced tablet porosity retards water penetration and delays or even inhibits the role of the super disintegrant. In addition, tablets when moistened, the super disintegrant expands and swells to cause rupture and complete the disintegration of the tablet. Hence, there was an indirect correlation between DT and WT. The decrease in the wetting time was indicating the enhancement of the tablet disintegration. The high absorption ratio indicating the large amount of water absorbed, which enhances disintegration of all tablets.

**Disintegration time**

**Measurement of DT by a modified apparatus**

The USP disintegration apparatus is not intended to measure accurately the DT of sublingual tablets because agitation is more vigorous. To overcome this problem, a modified dissolution test was employed.

In this test, the sinker (wire basket) was positioned in the vessel with the help of a hook in a way that the basket had only 10.5 ml of it.
Table 2: Physical evaluation of the stored and the fresh sublingual tablets

| The fresh tablets | F | H.* | %F* | Dt** | Dt*** | %C.U. | WT# | WAR## |
|-------------------|---|-----|-----|------|-------|-------|-----|-------|
| 1                 | 2.0±0.03 | 0.78 | 142±0.09 | 144±1.34 | 102.1±1.069 | 80.3±0.84 | 15.69±0.94 |
| 2                 | 2.4±0.25 | 0.35 | 172±0.08 | 191±0.33 | 100.2±0.65 | 90.6±0.41 | 17.35±0.13 |
| 3                 | 2.8±0.58 | 0.38 | 116±0.22 | 125±2.02 | 98.3±0.635 | 10.2±0.44 | 10.19±1.37 |
| 4                 | 4.2±0.07 | 0.34 | 228±1.22 | 231±0.99 | 99.7±0.273 | 240±0.40 | 4.84±1.21 |
| 5                 | 2.2±0.04 | 0.18 | 176±1.45 | 186±1.15 | 101.1±0.91 | 40.2±0.24 | 12.36±0.94 |
| 6                 | 2.2±0.59 | 0.42 | 54±2.02 | 61±0.05 | 100.7±0.92 | 29.1±0.48 | 18.87±1.01 |
| 7                 | 7.8±0.01 | 0.33 | >300 | 100±0.96 | 180.2±0.39 | 3.73±0.76 |
| 8                 | 1.7±0.025 | 0.17 | 206±0.61 | 216±0.02 | 101.9±0.46 | 14.8±0.23 | 25.24±0.66 |
| 9                 | 3.1±0.58 | 0.65 | 129±1.88 | 124±0.51 | 99.6±0.40 | 10.5±0.71 | 11.81±0.07 |
| 10                | 5.2±0.99 | 0.37 | >300 | 99.3±0.22 | 180.1±0.43 | 7.91±0.24 |
| 11                | 2.9±0.10 | 0.08 | 179±0.11 | 178±0.75 | 100.3±0.26 | 193.52±1.37 | 6.215±0.33 |
| 12                | 2.8±0.07 | 0.07 | 58±1.36 | 71±0.31 | 97.8±0.27 | 19.3±0.14 | 22.89±0.24 |

| The stored tablets | F | H.* | %F* | Dt** | Dt*** | %C.U. |
|-------------------|---|-----|-----|------|-------|-------|
| 1                 | 2.1±0.14 | 0.93 | 170±0.45 | 173±0.11 | 91.1±0.54 |
| 3                 | 2.3±0.08 | 0.52 | 139±0.09 | 154±0.09 | 98.6±0.57 |
| 6                 | 2.0±0.11 | 0.49 | 68±1.21 | 69±0.23 | 91.3±0.29 |
| 12                | 2.5±0.43 | 0.17 | 74±1.12 | 76±0.91 | 96.3±0.89 |

A ranking of the disintegration times indicates that: F7 and F10 have the highest DT as there was a direct correlation between hardness value and disintegration time. The greatest compact force leads to lower the tablets’ porosity. Lug¨ub¨ull and Leuenberger[34] confirmed that the water uptake must be the first step in the process of disintegration, which can be limited by the rate and extent of liquid absorption of the system.

Avicel type and diluents’ types had the significant effect on the DT (P<0.05). Not only there was no interaction between the effect of avicel type and the diluents used, but also it had no significant effect on the tablets’ disintegration (Data not shown) (P-value=0.0159, SE=1.844).

Only two formula namely F6 and F12 have disintegration times lesser than 1min. Avicel PH 102 has the smallest particle size in addition Avicel is a water insoluble binder. The high water solubility of other binders leads to increase in the viscosity of water, which brake or be a barrier formed against its penetration into the tablet resulting in delaying the DT.[35]

Except all formulae have the same type and concentration of the disintegrant (5% Ac Di Sol) but each formula has different DT. This was explained by the fact that the breakup of the tablets was not governed by the disintegration, but it depends on the rate at which the binder dissolved, since the latter was distributed across the particle surface.[36]

F6<F12 due to Avicel with Lactose as a diluent gives higher DT than with mannitol, These results are supported by the observation of some investigators who found an additive effect on the DT between lactose as fillers when it was combined with Avicel.[37]

F1 has low DT. The uneven surface of PVP with a folded structure increases the area subjected to the disintegration media.[38]

In-vivo oral disintegration time

Only Six formula namely F6, F12, F9, F3, F1 and F11 showed disintegration times less than 3 min, 61, 71, 124, 144 and 178 seconds respectively (European Pharmacopoeia, 2002, adopted the term orodis-persible tablet as a tablet to be placed in the mouth and disintegrated in less than 3 min).[39]

From the previous study tablets formula F1, F3, F6, F9, F11 and F12, have the best physical characters of all previous evaluation tests and hence, they were used for the following evaluation tests. HPLC method for the determination of (BH) in the presence of (Bu)

HPLC separation method explains; there was no interference between both drugs. Statistical evaluation found that the recovery% = 96.399% and 96.78% for BH and Bu respectively with a mean recovery 99.395% ± 0.47. The high recovery% value (95% -102%) indicated reduced the interference between both drugs.

The relative standard deviation was lesser than 2% in both cases. The LLOQ of the assay was calculated to be 0.05μg/ml. The assay showed acceptable precision and accuracy as precision ranged from 0.055 to 7.425 (C.V. %) and the accuracy ranged from 5.865 to 6.45 (relative error %). At all the results indicated that; the suitability of the HPLC method for the determination
Holistic analysis of BH and Bu respectively in unknown mixture samples. It also identified that the absence of possible chemical interaction between both drugs [Figure 2].

**In vitro dissolution study**

The best selected tablets prepared to use different formula were compared in this respect with the release of the market product (5mg Concor® tablets and 10 mg Buspare®). The values were calculated as the percentage of BH and Bu dissolve according to the predetermined drug content (Data not shown).

Ranking of dissolution rate of sublingual tablets for Bu was F6 > F3 > in 15 minutes, F11 in 20 minutes, F1 in 25 minutes. F12 showed the quickest dissolution rate at 10 minutes. Although for BH was F9 > F6 > F12 > F1 in 15 minutes, F11 in 20 minutes, and only F3 showed the quickest dissolution rate at 10 minutes.

Tablets containing Avicel and PVP showed the enhanced dissolution rate and this attribute to their rapid disintegration property (F1, F3, F6, F9 and F12). Tablets containing Avicel PH 102 (F6 and F12) showed a fast dissolution rate than that containing Avicel PH 101 (F3 and F9); as they have a smaller particle size and because Avicel PH-101 belongs to the cellulose I polymorphic form which formed stronger tablets.

PVP enhances the dissolution rate as it has the uneven surface with a folded structure, which increase the area subjected to the media. In addition, the presence of PVP K30 in F1 can probably be increased the wettability of drugs as, PVP K30 causes decreased the interfacial tension between the drug and the dissolution media.

All formula gave dissolution rate >70% after the dissolution during first 10 min. So, for assessment and comparison, the dissolution test was repeated for 10 minutes and aliquots were withdrawn from the release medium every 1 min and analyzed as mentioned before [Figure 3]. The dissolution rate of the drug during the first 10 minutes (DR-10) and the extent of dissolution after 10 minutes (DP10) were calculated.

In addition, the rate and extent of drug dissolution, defined;

Dissolution efficiency (DE%) at 60 minutes = \[ \frac{\int_0^t y \, dt}{y_{100} \cdot t} \times 100 \]

Where the integral is the area under the dissolution curve up to dissolution time t and y100 is the area of the rectangle described by 100% dissolution at the same time (data not shown).

There was a significant increase in both the DE% and the (DR-10) in all formula than that of the market products for both drugs. To be more precise, the highest percentage of drug dissolved after 3 minutes was from F6, F3, F12, and F1. Hence F1, F3, F6 and F12 were selected for storage study and in vitro permeation through the buccal mucosa.
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In vitro permeation through the buccal mucosa

As illustrated in Figure 4, it was noted that, F1, F3, F6 and F12 had diffusion percent 99.6%, 103.5%, 98.56% and 98.80% after 120 minutes for BH and for Bu 98.96%, 101.02%, 99.56% and 98.78% respectively. The PC for BH was 11.64, 9.77, 9.11, 9.17 cm/min and for Bu were 9.93, 8.14, 8.56 and 8.44 cm/min to formula F1, F3, F6 and F12 respectively.

In vitro, permeation study may offer a means of enhancing drug permeation through the buccal mucosa. Statistical analyses of the given results show no significant difference between either of each formula in both drugs even in diffused percent or in the PC.

Kinetic analysis of in vitro release and the in-vitro permeation data of BH and BuHCl from the prepared formula

The Kinetic analysis of the in-vitro release date of BH and BuHCl from different formulae was studied according to the determination coefficient (R2). It was found that different formulae had first order and 0.5 > n > 1; this indicates Non Fickian or Anomalous, However, the Kinetic analysis of the in-vitro permeation data of BH and Bu from different formulae was diffusion order and n<0.5, this indicates case, I or simple Fickian diffusion (Data not shown).

Hence, Drug release depends on two simultaneous rates’ processes, water migration into the matrix and the drug diffusion through continuously swelling strands.

Accelerated stability testing

None of the stored formulae (F1, F3, F6 and F12) showed any change in color or appearance throughout the storage period. On the other hand, some tablets of F12 showed faint discoloration by the 2nd week. The characteristics of the stored tablets are in the Table 2. The discoloration and the brown spots at the end of the storage period might be due to the presence of a high percentage of lactose in this formula. Reducing sugars, including lactose reported to undergo a non-enzymatic browning reaction with amines generally known as the Maillard reaction.[43]

According to Moore and Flanner for dissolution profiles to be considered similar, value of similarity factor (f2) should be as close as possible to 100 (range from 50 - 100, corresponds to 10% and 0% differences, respectively). The computed (f2) values were 51.96%, 56.222%, 75.277% and 69.05% for F1, F3, F6 and F12, respectively, indicating that the dissolution profiles of fresh and stored tablets could be considered similar, Figure 5.

The higher (f2) value in case of F6 and F12 indicated the higher the similarity between the dissolution profiles of fresh and stored tablets of these formulae. Except F12 showed marked decrease in the mechanical strength compared to F6. Statistical analysis approved that; all results (hardness, friability and disintegration time) had no significant difference. Hence, these formulas were physically stable after storage.

Based on all results of storage at 60°C and 75% R.H. for 3 weeks, and other physical characters F6 and F12 were the best selected formulae.

Bioavailability of BH and Bu in the selected formulae

The sublingual absorption of BH and Bu from different formulae compared to oral formulae is illustrated in Figure 6.

All formulae have Cmax higher than the Cmax of standard tablet (10mg Buspare®), as It was reported that; Bu is rapidly absorbed and undergoes extensive first-pass metabolism (90% hepatic metabolism). Following oral administration, 10 mg oral dose, lower peak plasma levels of unchanged drug, of 1 to 6 ng/ml was observed at 40 to 90 minutes.[44]

The presence of two peaks in the curve of tablet formulae 6 and 12 can be attributed to the presence of two absorption phases, namely; sublingual absorption that is characterized by a rapid onset of absorption and sharp decline, followed by intestinal absorption of the swallowed portion of the dose, which is characterized by a delayed onset. It was reported that the maximum effect of BH and Bu after oral administration occurred within 1.7-3h and 1-1.5 h respectively.

The results of the statistical analysis revealed that the
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Formulation had a significant effect on all the tested parameters at \( P < 0.05 \) (\( F_5, 17 n = 11.424, 6.469 \) and 43.890 for \( C_{p_{max}} \), \( T_{max} \) and \( AUC (0.\infty) \) of BH and \( F_5, 17 n = 194.95, 2.002 \) and 967.92 for \( C_{p_{max}} \), \( T_{max} \) and \( AUC (0.\infty) \) of Bu respectively). Based on these results, it was evident that the formulation exhibited the most significant effect on \( AUC (0-\infty) \). On the other hand, there was no significant difference between the subjects (rabbits) for all the tested parameters.

Multiple comparisons using the Tukey’s HSD test revealed that \( C_{p_{max}} \) of the 3 formulae extremely differed significantly from each other with the highest value observed for \( F_6 \) followed by \( F_{12} \) and finally, the market product. The relative bioavailability was 156.185%, 421.86% and 123.398%, 270.426% for BH and Bu of \( F_6 \) and \( F_{12} \) respectively compared to 5mg Concor® and 10mg Buspare®.

Based on all results, \( F_6 \) showed the highest \( C_{p_{max}} \) and relative bioavailability and the lowest \( T_{max} \) value when compared to any other formula, even with the oral commercial tablet (5mg Concor® and 10mg Buspare®). In addition, the maximum concentration of the drug that reached the blood was in the first 10 min which means a rapid onset of action and improved the extent of both drug’s absorption.

**Clinical study**

The physical examination of Patients did not show moderate dizziness considered possibly related to treatment and no trends or clinically relevant changes were noted. Substantially during the study, Patients were required to have a normal creatinine clearance >25 mL/min 1.73 m². Their hematology, serum chemistry, and urinalysis values were normal indicate the absence of possible toxicity appeared from that combination.

The rate and extent of DBP and SBP reduction were represented as the (MAP- AUC). The mean values for the area under the mean reduction of MAP-time curve AUC (0-6) were 79.79 ± 4.62, 11.39 ± 1.92 and 131.95 ± 11.06 after the oral administration of the market product 5 mg Concor®, 10 mg Buspare® and the sublingual administration of \( F_6 \) to the thirty subjects, respectively.

The sublingual tablets have SBP and DBP lesser than that of oral tablets, and the effect began from the first 10 min when compared that to oral dose \( T_{max} \) (DBP=2h , 4.25 h, 0.5 h and SBP=1.5h and 0.33 h for commercial formulae and \( F_6 \) respectively) as in Figure 7.

The results of the statistical analysis revealed that the formulation had a significant effect on the tested parameter MAP. (Using AUC as an expression of the rate and the extent at \( P < 0.05 \) (\( P \)-value = 0.351 and 0.212 for 5mg Concor-\( F_6 \) and 10 mg Buspare-\( F_6 \) respectively). Therefore, it was evident that, the prepared formula surpassed the market product concerning the both SBP and DBP.

The presence of Bu with BH in \( F_6 \) can be attributed to the
marked decline in DBP and SBP. That led to a reduction in the MAP. The main mode of action of BH is inhibition of chronic beta-1 stimulation-induced myocardial dysfunction. That led to decrease in SBP[46] and improve diastolic function in the left ventricle, which led to decrease in DBP[46]. Bu as reported by Shingala and Balaranan, 2005,[16] has an antihypertensive effect through aortic effect and vascular activity (DBP). Furthermore, it was reported that Bu reduced the SBP[17].

Conclusion

The suggested sublingual tablet (F6), which contains 5mg BH and 10mg BuHCl as a combination therapy showed higher bioavailability than commercial oral tablets. In addition, it considered a promising dosage form easy for handling of elderly patients suffering from stress hypertension and angina pectoris and improved the bioavailability of BuHCl. The significant findings presented here encourage further studies.

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Development and validation of spectrophotometric methods for simultaneous estimation of citicoline and piracetam in tablet dosage form

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ABSTRACT

Context: Citicoline (CN) and piracetam (PM) combination in tablet formulation is newly introduced in market. It is necessary to develop suitable quality control methods for rapid and accurate determination of these drugs.

Aim: The study aimed to develop the methods for simultaneous determination of CN and PM in combined dosage form.

Materials and Methods: The first method was developed by formation and solving simultaneous equations using 280.3 and 264.1 nm as two analytical wavelengths. Second method was absorbance ratio in which wavelengths selected were 256.6 nm as its absorptive point and 280.3 nm as λmax of CN. According to International Conference on Harmonization (ICH) norm, the parameters — linearity, precision, and accuracy were studied. The methods were validated statistically and by recovery studies.

Results: Both the drugs obeyed Beer-Lambert’s law at the selected wavelengths in concentration range of 5-13 µg/ml for CN and 10-22 µg/ml for PM. The percentage of CN and PM in marketed tablet formulation was found to be 99.006 ± 0.173 and 99.257 ± 0.613, respectively; by simultaneous equation method. For Q-Absorption ratio method the percentage of CN and PM was found to be 99.078 ± 0.158 and 99.708 ± 0.838, respectively.

Conclusions: The proposed methods were simple, reproducible, precise and robust. The methods can be successfully applied for routine analysis of tablets.

KEY WORDS: Absorbance ratio, citicoline, piracetam, recovery study, simultaneous spectrophotometry, validation

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The main disadvantage of this technique is its dependence on instrumental parameters like speed of scan and the slit width. No official procedure is given in pharmacopoeias for simultaneous spectrophotometric estimation of PM and CN. A liquid chromatography method for the determination of CT in injection, oral drops, and tablet dosage form,[8‑11] and spectrophotometric methods like colorimetry by complexation and UV-visible spectrophotometric methods using standard absorptivity value for determination of CT in pharmaceutical dosage form were also reported.[12‑16] Literature survey revealed high performance liquid chromatography (HPLC) methods for the estimation of PM in biological fluids.[17] Capillary electrophoresis, thin layer densitometric determination, and micellar electrokinetic chromatography methods were also developed for the estimation of PM in biological fluids.[18] In this present study, a successful attempt has been made to determine PM and CN in tablet dosage form by two methods based on simultaneous equation and Q-absorption ratio method. Both the methods were validated as per International Conference on Harmonization (ICH) norms.

Materials and Methods

Chemicals and reagents

Pure drug samples of PM and CN were obtained as gift sample from Akums Drugs and Pharmaceuticals, Mumbai. The combined dose PM (800 mg) + CN (500 mg) tablets (Strocit Plus) manufactured by Akums Drugs and Pharmaceuticals was procured from local medical shop. 0.1N methanolic HCl was used as solvent.

Instrumentation

Spectrophotometric analysis was performed on a double-beam Shimadzu 1800 UV-Visible spectrophotometer with data processing system, spectral bandwidth of 2 nm, wavelength accuracy ± 0.5 nm, and a pair of 10-cm matched quartz cells was used.

Preparation of stock and standard solutions for linearity

Stock solutions were prepared by dissolving PM and CN in 0.1 N methanolic HCl separately to obtain a concentration of 100 µg/ml of each compound. The standard solutions were prepared by dilution of the stock solutions in 0.1N methanolic HCl to reach concentration ranges of 10 µg/ml and 20 µg/ml for CN and PM, respectively.

Simultaneous equations method (method 1)

This method of analysis was based on the absorption of drugs CN and PM at the wavelength maxima of each other. The stock solutions of CN and PM were scanned separately in range of 200-350 nm to determine the wavelength of maximum absorption. From the overlain spectra [Figure 3], the wavelengths 280.3 and 264.1 nm was selected for CN and PM, respectively. For constructing a calibration curve, solutions of three series of different concentrations in range of 5-13 µg/ml for CN and 10-22 µg/ml for PM were prepared by appropriate dilution of stock solution with 0.1N methanolic HCl. Absorbance of these solutions were measured at 280.3 and 264.1 nm and calibration curves were plotted. The absorptivities (A%, 1 cm) of both the drugs at these two wavelengths were determined. These calculated values were the mean of six independent determinations. The concentration of the two drugs in mixture was calculated by using following equations:[19]
C\textsubscript{x} = \frac{A\textsubscript{y1}Q\textsubscript{y1} - A\textsubscript{y2}Q\textsubscript{y2}}{A\textsubscript{y1}Q\textsubscript{y1} - A\textsubscript{y2}Q\textsubscript{y2}} \quad (1)

C\textsubscript{y} = \frac{A\textsubscript{x1}Q\textsubscript{x1} - A\textsubscript{x2}Q\textsubscript{x2}}{A\textsubscript{x1}Q\textsubscript{x1} - A\textsubscript{x2}Q\textsubscript{x2}} \quad (2)

Where, C\textsubscript{x} and C\textsubscript{y} are the concentrations of CN and PM, respectively in mixture and in sample solutions. A\textsubscript{x} and A\textsubscript{y} are the absorbivities of sample at 280.3 and 264.1 nm, respectively; ax\textsubscript{1} and ax\textsubscript{2} are the absorbivities of CN at 280.3 and 264.1 nm, respectively; and ay\textsubscript{1} and ay\textsubscript{2} are the absorbivities of PM at 280.3 and 264.1 nm, respectively. The validity of the formed equations was checked by carrying out six different determinations.

**Q-absorption ratio method (method 2)**

This method is applicable to the drugs that obey Beer’s law at all wavelengths and the ratio of absorbance at any two wavelengths is a constant value, independent of concentration and path length. From the overlain spectra of CN and PM, two wavelengths 256.6 nm as isosbestic point and 280.3 nm (\lambda\textsubscript{max} of CN) were selected for formation of Q-absorption ratio equation. The calibration curves were determined in the concentration range 5-13 \(\mu\text{g/ml}\) for CN and 10-22 \(\mu\text{g/ml}\) for PM. The absorbivities (A\textsubscript{\lambda}, %, 1 cm) of both the drugs at both the wavelengths were determined. These calculated values were the mean of six independent determinations. The concentration of the individual components can be calculated by using the following equations: \[^{2b}\]

C\textsubscript{x} = \frac{Q\textsubscript{y1}Q\textsubscript{x2} - Q\textsubscript{y2}Q\textsubscript{x1}}{Q\textsubscript{y1}Q\textsubscript{x2} - Q\textsubscript{y2}Q\textsubscript{x1}} \quad (3)

C\textsubscript{y} = \frac{Q\textsubscript{x1}Q\textsubscript{y2} - Q\textsubscript{x2}Q\textsubscript{y1}}{Q\textsubscript{x1}Q\textsubscript{y2} - Q\textsubscript{x2}Q\textsubscript{y1}} \quad (4)

Where, C\textsubscript{x} and C\textsubscript{y} are the concentrations of CN and PM, respectively in mixture and in sample solutions. A\textsubscript{\lambda} and A\textsubscript{\lambda} are the absorbivities of sample at 280.3 and 256.6 nm, respectively. Q\textsubscript{\lambda} is the ratio of absorbance of sample at 256.6:280.3 nm, Q\textsubscript{\lambda} is the ratio of absorptivity of CN at 256.6:280.3 nm, \lambda\textsubscript{max} of CN. The absorbances of all standard solutions of both CN and PM were measured at these two wavelengths.

**Validation of methods**

**Linearity/range**

Five aliquots of each drug solution were taken from standard stock solution and transferred to 10 ml volumetric flask to get a final concentration of 5-13 \(\mu\text{g/ml}\) of CN and 10-22 \(\mu\text{g/ml}\) of PM using 0.1 N methanolic HCl. For simultaneous equation method, the absorbance of all standard solutions were measured at 280.3 and 256.6 nm, the calibration curves of absorbance vs concentration were plotted and correlation coefficient and regression line equations for both CN and PM were determined. For Q-Absorption ratio method, the wavelengths selected were 265.6 (isosbestic point) and 280.3 nm (\lambda\textsubscript{max} of CN). The absorbances of all standard solutions of both CN and PM were measured at these two wavelengths.

**Accuracy**

Accuracy of the developed method was confirmed by doing recovery study as per ICH norms at three different concentration levels 80, 100, and 120%. The absorbances were noted at respective wavelengths. The concentration of CN and PM were determined by the above methods in triplicate.

**Precision**

In intraday study, concentration of two drugs was calculated on the same day at an interval of 1 h. In interday study, concentration of drug contents were calculated on three different days. In both intra- and interday precision study, percentage relative standard deviation (% RSD) was calculated.

**Ruggedness**

Ruggedness of the two proposed methods was determined by carrying out analysis by three different analysts on different days.

**Results and Discussion**

In overlain spectra, CN and PM show prominent peaks at 280.3 and 264.1 nm. Hence, absorbances were measured at these two wavelengths for their determinations by simultaneous equation method. The linearity of CN and PM were found to be in the concentration range of 5-13 \(\mu\text{g/ml}\) and 10-22 \(\mu\text{g/ml}\). For Q-Absorption ratio method, two wavelengths 256.6 nm as isosbestic point and 280.3 nm (\lambda\textsubscript{max} of CN) were selected from the overlain spectra and the linearity was found to be 5-13 \(\mu\text{g/ml}\) and 10-22 \(\mu\text{g/ml}\), respectively. Calibration curves were plotted and regression analysis was carried out [Table 1]. The calibration curves indicated a good correlation between concentration and absorbance within the concentration range tested. The calibration curves are shown in Figures 4-9.
The percentage of CN and PM in marketed tablet formulation containing CN 500 mg and PM 800 mg were found to be 99.001 and 99.257%, respectively by simultaneous equation method. For Q-Absorption ratio method, the percentage of CN and PM were found to be 99.078 and 99.708%, respectively. The results obtained were comparable with the corresponding labeled amounts [Table 2].

Table 1: Regression analysis of calibration curve

| Method | Drug | Wavelength (nm) | Concentration (µg/ml) | Slope | Intercept | $R^2$ |
|--------|------|-----------------|-----------------------|-------|-----------|-------|
| 1      | CN   | 280.3           | 5-13                  | 0.087 | 0.023     | 0.999 |
|        |      | 256.6           | 10-22                 | 0.017 | 0.002     | 0.999 |
|        | PM   | 280.3           | 5-13                  | 0.014 | 0.0       | 0.999 |
|        |      | 264.1           | 10-22                 | 0.031 | 0.009     | 0.998 |
| 2      | CN   | 280.3           | 5-13                  | 0.087 | 0.023     | 0.999 |
|        |      | 264.1           | 10-22                 | 0.017 | 0.002     | 0.999 |

$R^2$: Correlation coefficient

Table 2: Results of analysis of piracetam and citicoline in tablets

| Method | Drug   | Label claim (mg) | % Estimated $\pm$ SD | % RSD |
|--------|--------|------------------|----------------------|-------|
| 1      | Citoline | 500              | 99.006±0.173         | 0.175 |
|        | Piracetam| 800              | 99.257±0.613         | 0.618 |
| 2      | Citoline | 500              | 99.078±0.156         | 0.160 |
|        | Piracetam| 800              | 99.708±0.838         | 0.840 |

$^*$Mean of six estimations; SD: Standard deviation, RSD: Relative SD

Figure 4: Calibration curve of citicoline at 280.3 nm

Figure 5: Calibration curve of citicoline at 264.1 nm

Figure 6: Calibration curve of citicoline at 256.6 nm

Figure 7: Calibration curve of piracetam at 280.3 nm

Figure 8: Calibration curve of piracetam at 264.1 nm

Figure 9: Calibration curve of piracetam at 256.6 nm
The accuracy of the methods was determined at 80, 100, and 120% level. The percent recovery by simultaneous equation method ranges from 99.55-99.933% and 99.167-100.112% for CN and PM, respectively. By Q-Absorption ratio method the ranges were found to be 98.73-99.797% and 99.283-99.981% for CN and PM, respectively.

The precision was confirmed by intermediate precision. The analysis of formulation was carried out for three times in the same day and on three successive days. The % RSD values for inter- and intraday analysis of formulation was found to be less than 2%.

The ruggedness was confirmed by different analysts. The % RSD values for different analysts were found to be less than 2%. Hence, the intermediate precision and ruggedness were confirmed. The results for intermediate precision and ruggedness are shown in Table 3.

**Conclusion**

Based on the results obtained, it can be concluded that the proposed UV-spectrophotometric methods (simultaneous equation method and Q-absorption ratio method) for simultaneous determination of CN and PM is rapid, economical, accurate, precise, and reproducible. The utility of the developed methods have been demonstrated by analysis of combined dose tablet formulation. Hence, the proposed method can be employed for quantitative determination of CN and PM in combined dose tablet formulation. Simultaneous equation method can be used to carry out dissolution study in combination tablet formulation.

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ABSTRACT

Purpose: Life-style and tobacco addiction is the major risk factors for cancer progression in all over the world. Knowledge gaps between tobacco addiction, obesity and cancer in Indian patients brought an interdisciplinary group of investigators together to discuss the present study. Patients and Methods: We calculated the body mass index (BMI) of all the patients (N = 927) who were diagnosed with cancer for its treatment. National Institutes of Health (NIH) criteria were used to categorize the patients. All the patients of this disease could be contacted in person to find out the history of the disease. Results: The frequency of addiction in urban cancer patient was found to be about 53.3% and in a rural area it was only 33.7%. Tobacco addiction was independently associated with younger age of cancer patient (odds ratio [OR] 2.242; 95% Confidence interval (CI) 1.653-3.042), obese (OR 7.433; 95% CI 3.746-14.750), overweight (OR 4.676; 95% CI 3.381-6.468) and advanced stage of cancer (OR 11.950; 95% CI 5.283-27.030). Conclusion: Tobacco consumption appears to be a major contributor to cancer in younger age with elevated BMI in India. Rapid changes in diet and life-style, increase in tobacco consumption appear to be strongly associated with the carcinoma in this middle-income country.

KEY WORDS: Addiction, body mass index, cancer, cancer stage, obesity, tobacco
Tobacco smoking is associated with 12% of the total burden of disease and injury and is the major single contributor to ill health in the Indigenous community, predominantly through ischemic heart disease, Chronic obstructive pulmonary disease (COPD), and lung cancer. Both obesity and use of tobacco are major risk factors for the chronic disease and premature death, both are disproportionately represented among lower socio-economic groups, both carry a social stigma and both are difficult to treat clinically.

A growing body of evidence also indicates that tobacco smoke is an independently associated with the insulin resistance and that the insulin resistant condition may contribute to the accelerated atherosclerosis that leads to excessive cardiovascular disease in the adult smokers. Tobacco smoke is clearly associated with dyslipidemias (increased low density lipoprotein (LDL) and decreased high density lipoprotein (HDL)), endothelial dysfunction, and a hypercoagulable state, all of which are also components of the metabolic syndrome. In addition, a dose-response relationship exists with cigarette smoking and the development of type II diabetes in adults. These findings suggest that because both, tobacco and the metabolic syndrome like obesity are individually associated with the insulin resistance. These all entities may be linked through this common pathophysiology and that overweight youth may be especially susceptible to the impact of tobacco smoke on carcinogenesis, considering that tobacco use and obesity both predispose to the constellation of risk factors seen in the carcinogenesis.

In order to determine the effect of active addiction to tobacco with markedly elevated BMI, age group, and disease advancement among cancer hospitalized patients, a review was made of medical records of individuals in whom an active addiction of tobacco was from 5 or more years in Department of Surgical Oncology, Sir Sundarlal Hospital, Banaras Hindu University, Varanasi (UP), India.

Patients and Methods

This consecutive cluster study included all patients who underwent treatment for invasive cancer at the Surgical Oncology Department, Sir Sundarlal Hospital Banaras Hindu University from January 2006 to April 2009. Exclusion criteria included patients <18 years of age, who received prior treatment in the form of chemotherapy/radiotherapy/surgery and patients who were mentally incapable of giving their own consent.

If the patient met appropriate criteria, we visited the patient before treatment to explain what the study was about and ask for patient participation. After proper consent from the patient, we conducted a 30-min interview with the patient. Data involving presentation, diagnosis, and staging were collected from office charts, hospital charts, and a face-to-face interview with the patient along with a questionnaire that included questions about height and weight, age, health-care and utilization, health habits, nature of food (Fibrous/junk-food/non-vegetarian), addiction, residence, and physical activity level. Diagnosis was defined by the histological presence of the carcinoma tissue biopsy, generally by pre-operative core biopsy.

Out of total N = 927 patients, 269 (29%) of oral cancer, 77 (8.3%) of lung cancer, 268 (28.9%) of gall bladder cancer, 143 (15.4%) of liver cancer and 170 (18.3%) of breast cancer patients.

Estimation of BMI

BMI was measured at the time of hospital admission for treatment and the criteria which were used to categorize patients are given in Table 1.

Distribution of patient according to specific addiction

Out of total N = 927 patients, 402 patients are addicted to substances that may be carcinogenic. These patients were further distributed according to their specific substance addiction and interactive substances used are given in Table 2.

Characterization of patients with socio-economic status and dietary habit

The patients that were included in the study belong to average middle class family of North Indian population. High and low consumption of fat was decided by preliminary evaluation of the diet habit. The process items were distributed with food habit (Fibrous/junk food/non-vegetarian) questionnaire to all members of an outdoor.

Statistical analysis

Demographic presentation and pathological factors were compared among the addicted and non-addicted groups. Two-tailed tests were used at all times, and statistical significance was set a priori at P < .05. Statistical analyses were performed with the SPSS for Windows 16.0. All univariate analyses used

Table 1: Classification of body mass index in adults

| Classifications | BMI (kg/m²) |
|----------------|------------|
| Underweight    | <18.5      |
| Normal range   | 18.5-24.9  |
| Overweight     | 25.0-29.9  |
| Obese          | ≥30.0      |

Table 2: Distribution of patient according to use of substances addicted

| Carcinogenic substances | Patients (N = 927) |
|-------------------------|-------------------|
|                         | Yes (%) | No (%) |
| Tobacco chewers         | 388 (41.9) | 539 (58.1) |
| Tobacco smokers         | 231 (24.9) | 696 (75.1) |
| Alcohol                 | 247 (26.6) | 680 (73.4) |
| Alcohol with tobacco    | 228 (24.6) | 699 (75.4) |
Student’s t-test or Chi-square tests, as appropriate. Logistic regressions were also performed to evaluate factors associated with the tobacco addiction.

**Results**

**Epidemiologic and demographic presentation of cancer patients**

Over the time period of the study, 927 patients underwent treatment for invasive carcinoma out of which 402 patients were addicted with some carcinogenic substances. The mean age of the whole patient was 48.76 ± 8.34 years but for addicted group the mean age was 47.24 ± 8.24 years for non-addicted patient. The distribution of addicted and non-addicted group is depicted in Figure 1, where we compared these groups with age group, and found that frequency of <45 years of age group is approximately half than, ≥45 years of age group in non-addicted group, but in addicted group frequency is approximately equal.

**Association of demographic presentation with the tobacco carcinogenesis**

Association between other demographic and pathological factor with the tobacco addiction are listed in Table 3. The preponderance of the study showed a significant positive association between addicted patient with age group, residence, stage of cancer, and diet nature.

The frequency of addiction in urban cancer patient is 53.3%, but in rural it is only 33.7%. When we compared these groups with cancer stage, we found a higher frequency of addicted patients were in their third (III) or fourth (IV) stage.

In the context of diet nature, we found 54.7% of addicted patient take high fat content diet and when we compared, this factor showed a significant association with the addiction of tobacco.

Logistic regression was performed to identify demographic and pathologic variables associated with the positive tobacco addiction. Age group < 45 years, higher BMI and advance cancer stage were formed to be independently associated with the tobacco addiction [Table 4].

**Relationship of demographic and pathological presentation with interactive addiction**

Out of total patients (N = 927), 228 (24.6%) patients were found addicted with both tobacco and alcohol. Association of demographic and pathological presentation with interactive addiction is listed in Table 5.

There was a significant association between interactive addiction persisted in multinomial logistic regression analysis, suggesting an independent association between the age group (odds ratio [OR] = 1.945, 95% CI = 1.407-2.688), overweight (OR = 2.090, 95% CI = 1.486-2.939), obese (OR = 2.287, 95% CI = 1.218-4.294), cancer stage III (OR = 3.160, 95% CI = 1.312-7.612), and cancer stage IV (OR = 7.704, 95% CI = 3.113-19.065).

**Discussion**

From a public health perspective, the effect of tobacco is

| Characteristics | Addicted (n=402) (%) | Non-addicted (n=525) (%) | P value |
|-----------------|----------------------|--------------------------|---------|
| Age group <45 years | 195 (53.3) | 171 (46.7) | <0.001 |
| ≥45 years | 207 (36.9) | 354 (63.1) | - |
| Residence Urban | 245 (53.1) | 216 (46.9) | <0.001 |
| Rural | 157 (33.7) | 309 (66.3) | - |
| Stage of cancer I | 9 (14.5) | 53 (85.5) | <0.001 |
| II | 87 (30.6) | 197 (69.4) | - |
| III | 187 (45.7) | 222 (54.3) | - |
| IV | 119 (69.2) | 54 (30.8) | - |
| Diet nature Low fat | 182 (39.4) | 280 (60.6) | <0.02 |
| High fat | 220 (47.3) | 245 (52.7) | - |

| Variable | Odds ratio | 95% confidence interval | P value |
|----------|------------|-------------------------|---------|
| Age group <45 years | 2.242 | 1.653-3.042 | <0.001 |
| ≥45 years | 1.0 | - | - |
| Body mass index Obese | 7.433 | 3.746-14.750 | <0.001 |
| Overweight | 4.676 | 3.381-6.468 | <0.001 |
| Underweight + normal weight | 1.0 | - | - |
| Cancer stage I | 1.0 | - | - |
| II | 3.040 | 1.386-6.664 | <0.01 |
| III | 6.110 | 2.827-13.207 | <0.001 |
| IV | 11.950 | 5.283-27.030 | <0.001 |
staggering, perhaps justifying its description as “the dangerous addiction of the developed world.” The detrimental cancer risk and prognosis associated with tobacco add’s to the impetus for public health policy, planning, and health education to successfully address the problem of tobacco addiction.

This study showed that the prevalence of tobacco consumption was significantly greater among victims dying due to malignancy and it is also affected by urbanization, sedentary life style, and high content of fat consumption that may lead to obesity [Table 3]. According to Indian Council of Medical Research estimates, the annual estimated mortality due to tobacco-related diseases varies between 630,000 and 1 million. At any point of time, there are about 374,000 cancer patients, 1.89 million patients with coronary artery disease, and 4.8 million patients with the chronic obstructive pulmonary disease, attributable to tobacco usage. The exact prevalence of tobacco consumption, particularly in relation to mortality, is not available from various parts of India.

Various methods of tobacco use such as cigarette, beedi, and hukka (water-pipe) smoking and tobacco chewing are common in different parts of India. The prevalence of smoking varies between 20% and 58% as reported in different studies from India.

Tobacco chewing, beedi, and hukka smoking appears to be more common in rural populations, whereas cigarette smoking and chewing of flavored tobacco are more common in the urban population of India. Among South-East Asian and Pacific Islander populations, chewable concoctions of combined betelnut and tobacco are popular, whereas for groups of Middle Eastern origin, the use of the hookah for smoking tobacco is a popular practice.

In this study, tobacco uses were ascertained in 43.4% of the cancer cases. Singletary and Gapstur, explored the association between tobacco use and breast cancer risk was observed that regardless of the type of addiction consumed. The prevalence of smoking varies between 20% and 58% as reported in different studies from India. Tobacco chewing, beedi, and hukka smoking appear to be more common in rural populations, whereas cigarette smoking and chewing of flavored tobacco are more common in the urban population of India. Tobacco consumption also damages the taste buds in the oral cavity, which may be a factor in the observed decreases in intake of fruits and vegetables resulting in poor vitamin C and beta-carotene status among tobacco users. These effects are known to predispose to cardiovascular diseases and cancer.

In this study, tobacco addiction was independently associated with a younger age of cancer patient (OR, 2.242, 95% CI, 1.653-3.042), obese (OR, 7.433; 95% CI, 3.746-14.750), overweight (OR, 4.676; 95% CI, 3.381-6.468) and advanced stage of cancer (OR, 11.950; 95% CI, 5.283-27.030) [Table 4]. The interactive addiction (tobacco and alcohol) followed the same pattern like tobacco addiction. Epidemiological studies carried out in India and abroad have shown that increased alcohol consumption is causally associated with cancers at various sites, mainly oral cavity, pharynx, larynx, and esophagus. Heavy alcohol drinkers are frequently heavy smokers as well. The adverse effects of smoking may be because of nicotine, carbon monoxide, and other chemicals, which may enhance carcinogens and free radical generation. Alcohol and tobacco use varied according to gender, age, and ethnicity, with men having higher rates of co-use than women. Younger people tended to have a higher prevalence of alcohol use disorders, nicotine dependence, and co-use.

A study of Panagiotakos et al., 2004 and Steenland et al., 1998 suggested that tobacco smoke is clearly associated with dyslipidemias (increased LDL and decreased HDL),[39] that clearly supports our result. Daniell demonstrated an independent association between lymph node metastases and obesity that was supported by Schapira et al., who showed that, in a study of 245 women, obese post-menopausal women were more likely to have axillary node metastases than normal weight patients.

Conversely, no association of tobacco smoking with thyroid cancer was found in two prospective cohort studies of men and women in the San Francisco Bay area, and of Canadian women, but the results were based on relatively small numbers of thyroid cancer patients. Negative associations between alcohol drinking and thyroid cancer have also been reported in some studies, but the results have been inconsistent.

There has been a marked increase in tobacco production and consumption in the last four decades in India, according to various national and world development reports. In developing countries such as India, China, Sri Lanka, and Brazil, massive occupational physical activity in lower social classes is defensive against morbidity and mortality due to circulatory diseases while they continue to have more deaths due to infections and poor nutritional status. Unfortunately, the impact of better education due to lack of health education, possibly, has not yet started in these countries.

Obesity leads to increased levels of fat tissue in the body that can store toxics and can serve as a continuous source of carcinogens. A recent study of Singh et al., explored, breast cancer risk was significantly associated with peri/post-menopausal status, residence, diet nature, and tobacco uses. He also concluded that

### Table 5: Logistic regression analysis of demographic and pathologic presentation with an interactive addiction of alcohol and tobacco

| Variable | Odds ratio | 95% confidence interval | P value |
|----------|------------|-------------------------|---------|
| Age group |            |                         |         |
| <45 years | 1.945      | 1.407-2.688             | <0.001  |
| ≥45 years | 1.0        |                        |         |
| Body mass index |   |                       |         |
| Obese     | 2.287      | 1.218-4.294             | 0.01    |
| Overweight| 2.090      | 1.486-2.939             | <0.001  |
| Underweight+normal weight | 1.0 |                        |         |
| Cancer stage |     |                       |         |
| I         | 1.0        |                        |         |
| II        | 1.422      | 0.567-3.565             | 0.453   |
| III       | 3.160      | 1.312-7.612             | 0.01    |
| IV        | 7.704      | 3.113-19.065            | <0.001  |
13.

Demographic factors, diet, tobacco, and obesity appear to be important causes of death due to cancers in both developed and developing countries. These populations need to learn the methods of prevention for which World Health Organization (WHO) and other International and national agencies are working hard independently. Estimated and projected mortality rates per 100,000 for India’s population by the WHO indicate that in the year 2000, all causes of deaths should be 876 in men and 790 in women which should decrease to 846 in men and 745 in women by the year 2015.

Conclusion

In brief, the findings of our study indicated that tobacco consumption has become a public health problem in India irrespective of social classes. The life-style behavior, elevated BMI, younger age group, and advanced cancer stage significantly associated with tobacco addiction. Mass scale and tobacco effect awareness through health promotion programs and medication should be implemented to diminish the risk factors responsible for cancer mortality risk in Northern India.

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Gastric ulcer is a common global problem with an increasing incidence and the prevalence in modern society. It is a kind of heterogeneous disorder causing due to imbalance between defensive and aggressive factors such as stress, exposure to bacterial infection, and use of the non-steroidal anti-inflammatory drugs [Figure 1]. Although the anti-secretory drugs, such as proton potassium ATPase (PPA) pump inhibitors-omeprazole, pantoprazole etc. and histamine H2-receptor blockers-ranitidine, famotidine scavenging of reactive oxygen species (ROS), inhibition of H⁺, K⁺-ATPase and Helicobacter pylori growth in the stomach, although the anti-secretory drugs, such as proton potassium ATPase (PPA) pump inhibitors-omeprazole, pantoprazole etc. and histamine H2-receptor blockers-ranitidine, famotidine 

**ABSTRACT**

**Aims:** The aim of this study was to evaluate the antioxidant activity and anti-acid property of *Acalypha wilkesiana* foliage extract. **Materials and Methods:** Hot and cold aqueous extracts were prepared from healthy leaves of *A. wilkesiana*. Free radical scavenging activity and H⁺, K⁺-ATPase inhibition activities of aqueous foliage extracts was screened by *in vitro* models. **Statistical Analysis Used:** All experiments were performed in triplicate and results are expressed as mean ± SEM. **Results:** *A. wilkesiana* hot aqueous extract (AWHE) showed significant antioxidants and free radical scavenging activity. Further, AWHE has shown a potent H⁺, K⁺-ATPase inhibitory activity (IC₅₀: 51.5 ± 0.28 µg/ml) when compare to standard proton pump inhibitor omeprazole (56.2 ± 0.64 µg/ml); however, latter activity is equal to *A. wilkesiana* cold aqueous extract (AWCE). Quantitative analysis of AWHE has revealed more content of phenols and flavonoids; this is found to be the reason for good antioxidant activity over AWCE. Molecular docking was carried out against H⁺, K⁺-ATPase enzyme crystal structure to validate the anti-acid activity of *A. wilkesiana* major phytochemicals. **Conclusions:** The present study indicates that the constituents of AWHE and AWCE have good antacid and free radical scavenging activity.

**KEY WORDS:** *Acalypha wilkesiana*, Anti-acid, antioxidant, gallic acid, gastric ulcer, molecular docking, polyphenols,
etc. are being used to control an acid secretion and acid related disorders.

Proton pump inhibitors (PPIs) are widely prescribed for acid-peptic disease. In general, safety of this class of drugs has been excellent. However, in the past several years, epidemiologic studies have indicated possible risks that are biologically plausible.\[5,6\] Hence, there is an urgent need of PPIs of plant or animal origin with the minimal side-effects since ulcer is the multi-step disease as the drugs available in the market fails to exert multi-step antiulcer effect with elevation of antioxidant levels, proton pump, and H. pylori growth inhibition. There is an increased interest in recent years to investigate antiulcer phytochemical drug with the multi-step activity.

The plant Acalypha wilkesiana commonly called as copper leaf, Joseph’s coat, and fire-dragon belongs to the sole genus Acalyphinae of the family Euphorbiaceae. The plant found all over the geographic area mainly used as an ornamental plant because of its striking foliage color. It consists of different terpenoides,\[7\] gallic acid, geranin, corilogan,\[8\] saponins, tannins, and glycosides.\[9\] The plant also has shown antibacterial, antifungal,\[10\] analgesic, antimalarial,\[10\] and antidiabetic activity.\[11\]

The present study was aimed to evaluate the antioxidant properties of hot and cold aqueous extracts of A. wilkesiana leaves and to relate their antioxidant power to antiulcer effect through proton pump inhibition.

**Materials and Methods**

**Chemicals and instruments**

Petroleum ether, ethanol, butylated hydroxyl anisole (BHA), 1-amino-2-naphthol-6-sulphonic acid (ANSA), perchloric acid, bovine serum albumin (BSA) were purchased from Merck (Germany). Gallic acid, catechin hydrate, quercetin, thio barbituric acid (TBA), and 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitro blue tetrazolium (NBT), nicotineamide adenine dinucleotide (NADH), phenazine methosulfate (PMS) were purchased from Sigma Aldrich (Poznan´, Poland). Trichloroacetic acid (TCA), Folin-Ciocalteau reagent was procured from Himedia (India). Water was purified on a Milli-Q system from Millipore (Millipore, Bedford, MA, USA). Absorbance was measured using PC based double beam spectrophotometer-2202 (Systronics, India). All the chemicals used were of analytical grade.

**Plant material and preparation of plant extract**

Healthy leaves of A. wilkesiana were collected from the orchard of bioscience complex, Kuvempu University, Shankaraghatta, Shivamogga, Karnataka, India. Plant was identified as A. wilkesiana by Dr. V. Krishna, Professor, Department of Biotechnology and Bioinformatics, Kuvempu University, Shankaraghatta. Healthy leaves were separated, shade dried, and pulverized mechanically (Sieve No. 10/44). 100 g of leaf powder was defatted using the petroleum ether in soxhlet apparatus. Defatted material was divided into two equal parts (50 g each). One part of material was used for hot aqueous extraction. Total 50 g of defatted leaf powder was taken in 500 ml millipore water and boiled for 60 min, cooled and filtered. The color solution was separated, dried, and referred as Acalypha wilkesiana hot aqueous extract (AWHE). Another part was used for cold aqueous extraction. Total 50 g of defatted leaf powder was mixed with 500 ml of millipore water mixed well and kept in the dark for an overnight. Then, filtered, dried, and referred as Acalypha wilkesiana cold aqueous extract (AWCE). These extracts were stored in desiccator to avoid oxidation and used for further studies.

**Quantitative analysis of crude extracts**

**Determination of total phenolic content**

Total phenolic content in crude extracts was measured by the Folin-Ciocalteau method using gallic acid as standard.\[12\] In brief, 2 ml of both AWHE and AWCE at different concentration (50-150 µg) in millipore water was mixed with 2.5 ml of Folin-Ciocalteau reagent (diluted 1:10 v/v) followed by the addition of 2 ml of Na₂CO₃ (7.5% w/v). Tubes were mixed and allowed to stand for 90 min at room temperature and absorbance of the sample was measured against the blank at 750 nm. Total phenolic content of the extract was expressed in terms of µg equivalent to gallic acid. All estimations were performed in triplicate and the results were averaged.

**Determination of total flavonoid**

Total flavonoids content of the extracts was determined according to modified method\[13\] using catechin as standard. Briefly, 2 ml of extracts at different concentrations (50-150 µg) in millipore water was mixed with 0.3 ml of 5% NaNO₂ and 0.3 ml of 10% AlCl₃ followed by the addition of 2 ml of 1 M NaOH after the incubation of the reaction mixture at room temperature for 6 min. Later, the volume in each test tube was made up to 5 ml by adding 2.4 ml of millipore water. Absorbance was measured against the blank at 510 nm. Total flavonoid content of the extract was expressed in terms of µg equivalent to catechin. Test was performed in triplicate and the results were averaged.

**Determination of antioxidant activity**

**Total antioxidant capacity**

Total antioxidant capacity of AWHE and AWCE was performed by phosphomolybdenum method as described by Prieto et al. \[14\] Both extracts at different concentrations (50-150 µg) in millipore water were taken in separate test tubes. To this, 5 ml of reagent mixture containing 4 mM ammonium molybdate, 0.6 M sulfuric
acid and 28 mM of sodium phosphate was added. Test tubes were kept for incubation at 95°C for 90 min. and allowed to cool. Absorbance of the content in each test tube was measured at 695 nm against blank. Antioxidant capacity of each extract is expressed as equivalents of ascorbic acid. Ascorbic acid equivalents are calculated using the standard graph of ascorbic acid. Test was performed in triplicate and the results were averaged.

**Total reductive capability**

Total reductive capacity of AWHE and AWCE was determined according to the method of Oyaiuzi. Both extracts at different concentrations (50-150 µg) in millipore water mixed with phosphate buffer (2.5 ml, 0.2 mol/L, pH 6.5) and potassium ferricyanide (2.5 ml, 1%). Then, the mixture was incubated at 50°C for 20 min. At the end of the incubation, TCA (2.5 ml, 10%) was added to the mixture, which was centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was collected and mixed with 2.5 ml of millipore water and ferric chloride (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank. Increased absorbance of the reaction mixture indicates increased reducing power. Test was performed in triplicate and the results were averaged. Reducing the capacity of each extract is expressed as equivalents of quercetin.

**DPPH radical scavenging activity**

AWHE and AWCE were screened for free radical scavenging activity by DPPH method of Braca et al. Free radical scavenging activity of the extracts was carried out based on the scavenging activity of stable DPPH. Extracts in millipore water at different concentration (5-15 µg) were added to each test tube and volume was made up to 2 ml using the millipore water. To this 3 ml of 0.004% DPPH in 95% ethanol was added and the mixtures were incubated at room temperature under dark condition for 30 min. The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm. Radical scavenging activity was calculated using the formula: % of radical scavenging activity = \( \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100 \), where \( A_{\text{control}} \) is the absorbance of the control sample and \( A_{\text{test}} \) is the absorbance of the test sample. The DPPH radical scavenging activity of BHA was also assayed for comparison. Test was performed in triplicate and the results were averaged.

**Superoxide radical scavenging assay**

Superoxide anion radical scavenging activity of AWHE and AWCE was measured according to the method of Nishikimi et al., 1972 with slight modification. All the solutions used in this experiment were prepared in phosphate buffer (pH 7.4). 1 ml of NBT (156 µM), 1 ml of NADH (468 µM) and 3 ml of extracts at different concentration (50-150 µg) were added. The reaction was started by adding 100 µl of PMS (60 µM) and the mixture was incubated at 25°C for 5 min. followed by the measurement of absorbance at 560 nm. Decreased absorbance of the reaction mixture indicated increased super oxide anion radical scavenging activity. The percentage inhibition was calculated from the formula, % of radical scavenging activity = \( \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100 \), where \( A_{\text{control}} \) is the absorbance of the control sample and \( A_{\text{test}} \) is the absorbance of the test sample. The Super oxide anion radical scavenging of ascorbic acid was also assayed for comparison. Test was performed in triplicate and the results were averaged.

**Lipid peroxidation inhibition assay**

TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm. 10% of egg homogenate was prepared using 0.15 M KCl. 0.5 ml of egg homogenate and 2 ml of AWHE and AWCE in millipore water at different concentrations (50-150 µg) were taken in test tubes. 100 µl of ferric chloride (0.2 mM) was added to each test tube and incubated at room temperature for 30 min. to induce lipid peroxidation. The reaction was stopped by adding 2 ml of ice-cold HCl (0.25 N) containing 15% TCA, 0.38% TBA, and 0.5% BHA. The content was mixed thoroughly and heated on boiling water bath for 60 min. Reaction mixture was cooled and centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was measured at 532 nm. Percentage of inhibition was calculated from the formula, % of inhibition = \( \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100 \), where \( A_{\text{control}} \) is the absorbance of the control sample and \( A_{\text{test}} \) is the absorbance of the test sample. Test was performed in triplicate and the results were averaged.

**Determination H+**, **K+**-ATPase inhibition

**Preparation of H+, K+-ATPase enzyme**

To prepare H+, K+-ATPase enzyme sample, fresh sheep stomach was obtained from a local slaughterhouse at Lakavalli, Karnataka, India. The stomach was cut open, the mucosa at gastric fundus was cut-off, and the inner layer was scraped out for parietal cells. Thus obtained cells were homogenized in 16 mM Tris buffer (pH 7.4) containing 10% Triton X-100 and centrifuged at 6000 g for 10 min. The supernatant (enzyme extract) was used to determine the H+, K+-ATPase inhibition. Protein content of the cell extract was determined according to Bradford’s method using the BSA as standard.

**Assessment of H+, K+-ATPase inhibition**

The reaction mixture containing 0.1 ml of enzyme extract (300 µg) and plant extract at different concentrations was pre-incubated for 60 min at 37°C. The reaction was initiated by adding substrate 2 mM ATP (200 µL), in addition to this 2 mM MgCl2 (200 µL) and 10 mM KCl (200 µL) was added. After 30 min of incubation at 37°C, the reaction was stopped by the addition of assay mixture containing 4.5% ammonium molybdate and 60% perchloric acid followed by centrifugation at 2000 g for 10 min and inorganic phosphate released was measured spectrophotometrically at 660 nm by following Fiske-Subbarow method. Briefly, to the 1 ml of supernatant 4 ml of millipore water, 1 ml of 2.5% ammonium molybdate, 0.4 ml of ANSA was added and allowed to stand for 10 min at room temperature. Absorbance of released inorganic phosphate was measured at 660 nm. Enzyme activity was calculated as micromoles of Pi released per hour at various doses (0-100 µg) of AWCE and AWHE. Results were compared with the known.
antiulcer PPA inhibitor drug omeprazole and expressed as Mean ± SEM.

Percentage of enzyme inhibition was calculated by using the formula;

\[
\text{Percentage of inhibition} = \left(\frac{\text{Activity (control)} - \text{Activity (test)}}{\text{Activity (control)}}\right) \times 100.
\]

**Molecular docking studies**

As reports of earlier investigator suggests, A. wilkesiana consists of a major pharmacologically active antioxidant molecule signatures such as gallic acid, corilagin, geranin, quercetin, kaempferol, artemetin, luteolin, and vitexicarpin.\(^{[8,24]}\) Molecular docking was performed for these reported phytochemicals of A. wilkesiana against PPA to substantiate the in vitro PPA inhibition by extract and their eventual comparison with standard omeprazole, being used in the treatment of gastric acid-secretory related disorder. A Lamarckian genetic algorithm method implemented in the program AutoDock 4.2 was employed to determine the orientation of phytochemicals with the active site of PPA. The ligand molecules [Table 1] were designed and the structure was analyzed by using ChemDraw Ultra 6.0. 3D coordinates prepared using PRODRG (http://davapc1.bioch.dundee.ac.uk/prodrg/) server.\(^{[25]}\) The protein structure file PDB ID: 2XZB [Figure 2] was taken from PDB (www.rcsb.org/pdb) and edited by removing the heteroatoms with simultaneous adding of C terminal oxygen.\(^{[26]}\) For docking calculations, Gasteiger-Marsili partial charges\(^{[27]}\) were assigned to the ligands, non-polar hydrogen atoms were merged and all torsions were allowed to rotate during docking. Active pockets were identified and ligplot of PDB Sum provided in the external links of PDB for the proteins was downloaded from PDB. Computed atlas of surface topography of proteins server was used to cross-check the active pockets on the target protein molecules. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for energy minimization using default parameters.

**Statistical analysis**

Results are expressed as Mean ± SEM of three parallel measurements. Statistical analysis was carried out by using the SPSS 17.0.

**Results**

Many studies have shown the potential benefits of antioxidants from plant sources. The present study showing the antioxidant activity and PPA inhibition property of foliage extracts of A. wilkesiana.

**Qualitative analysis of crude extracts**

The AWHE and AWCE was extracted from the foliage part of A. wilkesiana and the preliminary phytochemical screening indicates the presence of flavonoids, glycosides, triterpenoids, saponins, and tannins [Table 2].

**Quantitative analysis of crude extracts**

Total phenolic contents of AWHE and AWCE are expressed as µg equivalent to gallic acid. Among the extracts, AWHE has a significant amount of phenolic content (0.888 g/g of extract) when compare to AWCE (0.666 g/g of extract).

Flavonoids are important secondary plant metabolites, which increase with plant stress.\(^{[28,29]}\) The total flavonoid content of AWHE and AWCE was found to be 0.427 and 0.424 g/g of dry extract respectively.

**Determination of antioxidant activity**

**Total antioxidant capacity**

Total antioxidant capacity of both extracts was performed by phosphomolybdenum method as described by earlier.\(^{[16]}\) Antioxidant capacities are expressed as equivalents of ascorbic acid. Both extracts had shown significant antioxidant activity as equivalents to ascorbic acid. The results of total antioxidant activity were presented in Figure 3.

**Total reductive capacity**

The reductive ability of extracts was determined according to the method of Oyaizu, 1986.\(^{[17]}\) Quercetin was used as a standard to compare the activity of extracts. Among the extracts, AWCE showed excellent activity, whereas AWHE showed moderate activity [Figure 3b].

**DPPH radical scavenging activity**

Both extracts were screened for free radical scavenging activity using the DPPH method and it is based on the measurement of the reducing ability of antioxidants on DPPH free radical.\(^{[28,29]}\) Figure 3c represents the percentage of DPPH scavenging activity of AWHE, AWCE and BHT. AWHE and AWCE showed significant radical scavenging activity (79.9 ± 0.89 and 78.8 ± 0.52%) at 15 µg when compare to similar concentration of BHT (64.52 ± 0.56%). The variation exhibited in DPPH scavenging capacity was attributed to the synergistic contribution of mixture of more phenolics, flavonoids, and other molecules in AWHE and AWCE.

**Figure 2: Structure of H⁺, K⁺-ATPase (PDB ID: 2XZB)**
Table 1: Major phytoconstituents of *Acalypha wilkesiana*

| Phytochemical | PUBCHEM ID | Structure | Reference          |
|---------------|------------|-----------|--------------------|
| Artemetin     | 5320351    | ![Structure](image1.png) | Eugene et al., 2010 |
| Corilagin     | 5089683    | ![Structure](image2.png) | Adesina et al., 2000 |
| Gallic acid   | 370        | ![Structure](image3.png) | Adesina et al., 2000 |
| Geranin       | 44566353   | ![Structure](image4.png) | Adesina et al., 2000 |
| Kaempferol    | 5280863    | ![Structure](image5.png) | Adesina et al., 2000 |

Contd....
Superoxide radical scavenging assay

AWHE and AWCE were screened for superoxide radical scavenging activity using ascorbic acid as a standard. Superoxide radical is known to be very harmful as a precursor of more reactive oxidative species that have potential to react with biological macromolecules and thereby inducing tissue damage.\(^{[50]}\) Superoxide radical scavenging activity of extracts is presented in Figure 3d. Both the extracts showed significant dose dependent activity. At 150 µg, AWHE and AWCE showed 73.2 ± 1.02 and 68.6 ± 0.78% of inhibition respectively when compare to BHT (71.0 ± 0.25%).

Lipid peroxidation inhibition assay

The results of the AWHE and AWCE to prevent lipid peroxidation were shown in Figure 3e. At 150 µg, AWHE and AWCE showed 72.9% and 70.2% of inhibition respectively.

Table 1: Contd....

| Phytochemical | PUBCHEM ID | Structure | Reference |
|---------------|------------|-----------|-----------|
| Lutein        | 6433159    | ![Image](https://example.com/lutein.png) | Eugene et al., 2010 |
| Luteolin      | 5280445    | ![Image](https://example.com/luteolin.png) | Eugene et al., 2010 |
| Quercetin     | 5280343    | ![Image](https://example.com/quercetin.png) | Adesina et al., 2000 |
| Vitexicarpin  | 5315263    | ![Image](https://example.com/vitexicarpin.png) | Eugene et al., 2010 |

Table 2: Phytochemical screening of extracts of *Acalypha wilkesiana*

| Test          | Procedure                        | AWHE | AWCE |
|---------------|----------------------------------|------|------|
| Alkaloids     | Sample +                         | −    | −    |
| Sterols       | Liebermann test                  | −    | −    |
| Flavonoids    | Zn-HCl acid reduction test       | +    | +    |
| Glycosides    | Sample + Anthrone + H₂SO₄ + Heat | −    | −    |
| Terpenoids    | Sample + chloroform + conc.H₂SO₄ | +    | +    |
| Tannins       | Sample + lead acetate + water    | +    | +    |
| Quinones      | Sample + conc.H₂SO₄              | +    | +    |
| Saponins      | Sample + water + Shake well      | −    | −    |
| Coumarins     | Sample + 10% NaOH                | +    | +    |
| Phenolics     | Sample + FeCl₃ solution          | +    | +    |

+: Present, −: Absent, AWHE: *Acalypha wilkesiana* hot aqueous extract, AWCE: *A. wilkesiana* cold aqueous extract
Lipid peroxidation, a well-established mechanism of cellular injury in plants and animals is used as an indicator of oxidative stress in cells and tissues. Polyunsaturated fatty acid peroxides generate MDA and the measurement of MDA has been used as an indicator of lipid peroxidation by means of derivatizing with TBA at high temperature and acidic condition.  

**H⁺, K⁺-ATPase inhibition study**

Figure 4 suggests the PPA inhibition activity of AWCE and AWHE as well as omeprazole as the reference standard. Both extracts showed dose depended activity. At a concentration of 100 µg/ml AWHE, AWCE, and omeprazole showed 88.7 ± 1.54, 79.99 ± 1.43 and 86.7 ± 0.56% of inhibition respectively. The concentration required to inhibit 50% of H⁺, K⁺-ATPase activity is designated as IC₅₀, thereby AWHE and AWCE showed IC₅₀
values of 51.5 ± 0.28 and 56.3 ± 0.89 µg/ml respectively, in comparison with omeprazole IC$_{50}$ value of 56.2 ± 0.64 µg/ml.

Validation of H$^+$, K$^+$-ATPase inhibition through molecular docking studies

Automated docking was used to assess the orientation of inhibitors bound with the active pockets of PPA. In general, binding of inhibitor to enzyme leads to change in the confirmation of enzyme and consequently arrest its activity. Figure 5 clearly indicates, the interaction of phytoconstituents of \textit{A. wilkesiana} with the active pocket of PPA. Interestingly, all the phytoconstituents showed minimum binding energy with PPA through non-covalent interaction [Table 3]. The \textit{in silico} observation confirm the \textit{in vitro} PPA inhibitory activity of AWHE and AWCE. The activity of extracts is due to stable and active synergistic role of major phytoconstituents present in \textit{A. wilkesiana}.

Table 3: Molecular docking studies of phytoconstituents of \textit{A. wilkesiana}

| Molecule      | Binding energy (kJ mol$^{-1}$) | Ligand efficiency | RMS | No. of H bond | Bonding                              | Bond length (Å) |
|---------------|--------------------------------|-------------------|-----|--------------|--------------------------------------|-----------------|
| Artemetin     | −6.43                          | −0.23             | 0.0 | 1            | DRG:OAM::PPA:B:TYR44:HH               | 2.193           |
| Corilagin     | −8.90                          | −0.2              | 0.0 | 5            | DRG:HAG::PPA:B:TYR40:OH               | 1.901           |
|               |                                 |                   |     |              | DRG:OA::PPA:A:GLY855:HN               | 1.962           |
|               |                                 |                   |     |              | DRG:HBQ::PPA:A:GLU847:O               | 2.088           |
|               |                                 |                   |     |              | DRG:HAI::PPA:A:ILE850:OH              | 2.222           |
|               |                                 |                   |     |              | DRG:HAH::PPA:A:SER851:O               | 2.238           |
| Gallic acid   | −8.37                          | −0.26             | 0.02| 3            | DRG:HAE::PPA:A:ILE850:OH              | 1.822           |
|               |                                 |                   |     |              | DRG:OAC::PPA:A:GLY855:HN              | 2.219           |
|               |                                 |                   |     |              | DRG:OAA::PPA:B:TYR44:HH               | 2.192           |
|               |                                 |                   |     |              | DRG:HAD::PPA:B:TYR40:OH               | 2.128           |
| Geranin       | −8.37                          | −0.21             | 0.0 | 1            | DRG:OBN::PPA:A:GLY855:HN              | 1.879           |
| Kaempferol    | −6.51                          | −0.28             | 0.0 | 1            | DRG:OAA::PPA:A:GLY855:HN              | 2.209           |
| Lutein        | −3.75                          | −0.09             | 0.0 | 1            | DRG:OB::PPA:B:TYR44:HH                | 2.084           |
| Luteolin      | −6.04                          | −0.29             | 0.0 | 1            | DRG:OAC::PPA:B:TYR40:HH               | 2.198           |
| Quercetin     | −6.12                          | −0.28             | 0.0 | 2            | DRG:OAB::PPA:A:THR788:HN              | 1.883           |
| Quercetin     | −5.61                          | −0.21             | 0.0 | 1            | DRG:HBB::PPA:A:GLU847:O               | 2.225           |
| Omeprazole    | −5.19                          | −0.22             | 0.0 | ---          | ---                                  | ---             |

RMS: Root mean square

Discussion

Metabolic pathway of the human body is continuously exposures to several degradative stresses, such as ROS and free radicals. These reactive species extensively cause the oxidative damage to the biomolecules nucleic acids, proteins and lipids[31] and contribute to the pathogenesis of oxidative stress related diseases such as cancer, ageing, heart failure, ulcer etc.[32] Antioxidants are considered as possible protective agents against oxidative damage of the human body. Consequently, there is a growing interest in the substances exhibiting antioxidant properties that are supplied to human and animal organisms. In that concern, natural antioxidants have become one of the major areas of scientific research.[33,34]
The effective antioxidants from natural sources are the only alternative to synthetic molecules in scavenging the free radicals during the prevention of accelerating pathogenicity. The present study demonstrates the in vitro free radical scavenging activity and PPA inhibition activity of aqueous extracts of A. wilkesiana. The study was further validated by in silico approach.

In this study, A. wilkesiana hot and cold leaf extracts were prepared and preliminary phytochemical investigation indicates the presence of various metabolites having good antioxidant activity such as phenolics, flavonoids, tannins, terpenes etc. In general, phenol and flavonoid content of plant plays a significant role in scavenging free radicals by acting as an electron donor and serves as antioxidants with considerable health benefits. Interestingly, both AWHE and AWCE consist of the enormous amount of phenol and flavonoid content and showed significant free radical scavenging activity at a minimum concentration and the result is in agreement with the presence of various antioxidant molecules in A. wilkesiana has revealed by Adesina et al. and Eugene et al. of which, AWHE showed significant antioxidant, reducing and free radical scavenging activity compare to AWCE since in hot condition most of the secondary metabolites dissolve readily into the water.

Along with free radical scavenging activity and antioxidant activity, H⁺, K⁺-ATPase inhibition by AWHE and AWCE was also assessed. The human stomach is found with the numerous gastric pits from which acid get secret. One of the cells, which lining the gastric pits is parietal cell, which is responsible for the acidification of stomach. PPA, the proton pump present in parietal cell is responsible for acid, which locates in the gastric membrane vesicle and actively transports protons into the lumen of stomach with the hydrolysis of the cytoplasmic ATP. Hyper secretion of this enzyme leads to acidity and ulcer. Therefore, this regulatory enzyme has found to be a pharmacological target to treat ulcer. Presently, ulcer is treated by providing allopathic PPIs, antioxidants, Hᵢ⁺-receptor antagonist etc., but these are associated with side effects. Hence, there is an urgent need of PPI of plant origin. Consequently, our present study is on in vitro PPA inhibition activity of foliage extract of A. wilkesiana. The study was evaluated by using sheep parietal cells where meprazole was used as positive standard. AWHE showed a high degree of PPA inhibition. To further validate and for better understanding of PPA inhibition activity by A. wilkesiana, an in silico molecular docking approach was done with major reported phytoconstituents present in A. wilkesiana against the crystal structure of PPA. All the molecules showed good interaction with minimum binding energy in the enzyme active pocket through non-competitive interaction.

Analysis of in-vitro and in silico results indicates that AWHE and AWCE has good free radical scavenging activity by virtue of total antioxidant property, pertaining to this it also showed significant PPA inhibition activity indicating its presumed anti-gastric ulcer property.

Furthermore, detailed studies on the isolation and characterization of the plant extract as well as in vivo assays will be necessary in discovering new biological antioxidants and anti-gastric ulcer drug.

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In recent years, lay public are progressively more and more concerned about their health-care by active involvement in decision making. The prescribers at times require giving additional information for prescribed medicines. Patients obtained medicine information through various sources, more commonly from internet because of its easy access and availability in many cultures. On the contrary, it is difficult to rely on the information obtained through internet and, therefore, validity of the internet information is questionable.

Adverse drug reactions (ADRs) have a major impact on public health by imposing a considerable economic burden on society and the already-stretched health-care systems.\(^1,2\) Post marketing surveillance of drugs is very important in investigating and controlling the risks associated with drugs once they are accessible for the use of the general population.\(^1,2\) The involvement of health-care professionals towards ADR reporting databases is a requirement. This supports continuous ascertainment of the benefit-risk ratio of some drugs as well as contributory toward the detection of unexpected and unusual ADRs, which were previously undetected during the initial evaluation of a drug.\(^1,2\) Despite these merits, under-reporting remains a major draw-back of spontaneous reporting. It is estimated that only 6–10% of all ADRs are reported.\(^1,2\)
In Malaysia, Malaysian Adverse Drug Reactions Advisory Committee (MADRAC) performs the function of monitoring safety profiles of drugs registered for use in Malaysia. Under this monitoring program, all the received reports are screened by MADRAC before being proposed to the World Health Organization’s Collaborating Center for Drug Safety Program in Uppsala, Sweden for inclusion in its ADR database. The primary functions of MADRAC include promoting ADR reporting in Malaysia, providing information and advice to the drug control authority so that regulatory action can be taken based on the ADRs received in Malaysia and internationally, and thus render information to doctors, pharmacists and other health-care professionals on ADRs. The challenge faced by MADRAC is, in principal, getting marketing authorization holders, health-care professionals, and consumers for reporting ADRs.

Globally, pharmacovigilance studies are the need of the hour for invariable monitoring of undesirable effects in hospital and community settings. The involvement of every stakeholder regarding reporting of ADR is of prime importance. Doctors, pharmacists, nurses, future practitioners and as well as lay public are all informed for reporting ADRs, which in turn contributes to increased medication safety for the population.

In the backdrop of this, we decided to conduct a pilot study on the knowledge of the general population toward ADR and their reporting system.

Methods

Study plan

A descriptive study was conducted to determine the knowledge of the general public in Penang toward ADRs identification and reporting. Interviews using a structured questionnaire were conducted over a week period in August 2009.

Study instrument

The study instrument was a questionnaire with 15 survey items. It was formatted as a paper-based survey and was divided into two sections; demographic characteristics and ADRs reporting knowledge.

Demographic characteristics consisted of five items; age, sex, race, educational background, and marital status. The section two comprised of 10 items with four items covered the medication and side-effect history; five items evaluate the understanding and response to ADR situation, and one item related to the history of reporting ADRs.

The questionnaire was subjected to face validity and content validity by sending to pharmacy academics who were involved in delivering the curricular contents related to pharmacovigilance. Their feedbacks were incorporated. The instrument was then administered to 10 males and females living in Penang. The data were then subjected to Statistical Package for the Social Sciences (SPSS) for reliability coefficient. A Cronbach’s alpha of 0.71 was computed.

Study subjects and site

This study recruited proportionately large convenience sample of the general public in Penang. Respondents easily approachable and willing to participate from all the ethnicities of not less than 18 years old, with different marital statuses and educational background were recruited in the survey. The survey was conveniently conducted at shopping complexes, bus stations, and medical centers covering all area of Penang. The sample size was calculated by Raosoft® online sample calculator with the margin of error 5%, and confidence level of 95% and therefore, the recommended sample size was calculated to be 368. A total of 334 respondents participated in the current study.

Data analysis

Data collected from the questionnaire was entered into a SPSS, version 17.0. Chi-square test is generally used to see the association between the variables. If the assumption for the Chi-square test does not seem to be justified that is expected cell number was lower than five in the contingency table, than Fisher’s exact test will be used to evaluate the association between dependent and independent variables. In the current study, Fisher’s exact test is applied and a value of $P < 0.05$ is considered to be significant.

Results

The total number of the general public in Penang responded to the survey was 334. Slightly more than half of the respondents were in the age group of 18-25 years (53.6%; n = 179). Majority of the respondents were found to be either diploma holders or Sijil Tinggi Persekolahan Malaysia, which is a Malaysian Higher Secondary School Certificate. For detailed demographics please see Table 1.

The first question inquired about the status of respondents being ever taken any medications or never takes any medications. No significant difference was observed in terms of being taken any medications (56.6%, $n = 189$) or never takes any medications (43.4%, $n = 145$).

When asked about the sources of their medication majority of them reported medical doctor (85.6%), whereas small number (34.7%) reported community pharmacists as sources of medications. Only a very few number of respondents (5.4%) reported traditional medicine practitioners as their source of medication. A high percentage of the respondents who are in the younger age group 18-25 years prefer to choose medical doctor rather than a pharmacist for their sources of medication. Respondents from the age group of 26-35 years prefer to get their medications from medical doctors, pharmacists, and traditional medicine practitioners. Interestingly, respondents from the age group of late 30 and above mostly choose traditional medicine practitioners as their source of

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Interestingly respondents with Sijil Pelajaran Malaysia (SPM) as educational background were found to be keen in choosing traditional medicine practitioners as their sources of medication.

The respondents were also asked about the reporting of side-effects of the drugs. Two-third of the respondent (66.8%, \( n = 223 \)) have reported side-effects to their medical doctors. The results were significant for comparing in between age groups and reporting to the medical doctor (\( P = 0.038 \)).

Interestingly, it was noted that three-quarter of the respondents (77.2%) get their information about the side-effects of drugs from physicians, followed by pharmacist (44.6%). For detailed results please refer to Tables 2 and 3.

The responses on awareness of respondents towards consumer ADR center set up by the ministry of health seem to be quite interesting. More than half of the respondents (65.6%, \( n = 219 \)) reported unawareness about the existence of ADR center set up by the Ministry of Health. This showed statistical significance with respect to age (\( P = 0.039 \)).

The respondents then were asked about their opinion on the safety of the marketed drugs.

Majority of the respondents believed that the drugs marketed were not safe (81.1%, \( n = 271 \)). Among the respondents who felt that not all drugs that were marketed were safe, 52.4% were 18-25 years old and 39.8% hold bachelor degree. For detailed results refer to Table 4.

**Discussion**

This pilot study encompasses the knowledge and opinion toward ADR reporting and its system among the general public.

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**Table 1: Socio-demographic data for study participants \( (N = 334) \)**

| Characteristics               | \( N \) | (%)  |
|-------------------------------|--------|------|
| Age                           |        |      |
| 18-25                         | 179    | (53.6)|
| 26-35                         | 77     | (23.1)|
| 36-40                         | 27     | (8.1 )|
| 41 and above                  | 51     | (15.3)|
| Gender                        |        |      |
| Male                          | 171    | (51.2)|
| Female                        | 163    | (48.8)|
| Race                          |        |      |
| Malay                         | 199    | (59.6)|
| Chinese                       | 85     | (25.4)|
| Indian                        | 43     | (12.9)|
| Others                        | 7      | (2.1 )|
| Educational level             |        |      |
| SPM                           | 78     | (23.4)|
| Diploma/STPM                  | 84     | (25.1)|
| Bachelor degree               | 132    | (39.5)|
| Post-graduate degree          | 40     | (12.0)|
| Marital status                |        |      |
| Married                       | 120    | (35.9)|
| Single                        | 214    | (64.1)|
| History of taken medications  |        |      |
| Yes                           | 189    | (56.6)|
| No                            | 145    | (43.4)|

SPM: Sijil Pelajaran Malaysia, STPM: Sijil Tinggi Persekolahan Malaysia

**Table 2: Awareness towards adverse drug reactions ADRs**

| Statement                                                                 | Responses | Fisher exact test \( P \) values | \( P \) values |
|---------------------------------------------------------------------------|-----------|-----------------------------------|---------------|
| Respondent aware of the existence of consumer adverse drug reaction ADR   | 115       | 219                               | 0.039 0.488   |
| center set up by the ministry of health                                   |           |                                   |               |
| All drugs that have been marketed are safe                                | 63        | 271                               | 0.146 0.668   |
| Pharmacists or doctor tell the side-effects of medications that are taking| 241       | 93                                | 0.968 0.976   |
| It is necessary for the health-care provider to provide the information about the medication that has been prescribed | 321       | 13                                | 0.611 0.048   |
| Experienced any side-effects due to the medications that have been taken  | 124       | 210                               | 0.001 0.870   |
| ADR: Adverse drug reaction                                                | 37.1      | 62.9                              |               |

**Table 3: Sources of information about side-effects**

| Statement                                                                 | Responses | Fisher exact test \( P \) values | \( P \) values |
|---------------------------------------------------------------------------|-----------|-----------------------------------|---------------|
| Respondents get the information regarding the side-effect of the drug taken from the physician | 258       | 76                                | 0.776 0.068   |
| Respondents get the information regarding the side-effect of the drug taken from the pharmacist | 149       | 185                               | 0.243 0.036   |
| Respondents get the information regarding the side-effect of the drug taken from the relative or friends | 87        | 247                               | 0.020 0.926   |
| Respondents get the information regarding the side-effect of the drug taken from the newspaper | 96        | 238                               | 0.003 0.692   |
| Respondents get the information regarding the side-effect of the drug taken from the television or radio | 61        | 273                               | 0.478 0.566   |
| Respondents get the information regarding the side-effect of the drug taken from the other sources | 38        | 296                               | 0.364 0.126   |

**Table 4: Sources of information**

| Statement                                                                 | Responses | Fisher exact test \( P \) values | \( P \) values |
|---------------------------------------------------------------------------|-----------|-----------------------------------|---------------|
| Respondent normally get their medications from physician                 | 286       | 48                                | 0.377 0.118   |
| Respondent normally get their medications from pharmacy                  | 116       | 218                               | 0.713 0.829   |
| Respondent normally get their medications from traditional medicine practitioner | 34.7     | 65.3                             |               |
| Respondent normally get their medications from traditional medicine practitioner | 18       | 316                               | 0.001 0.014   |

(77.2%) get their information about the side-effects of drugs from physicians, followed by pharmacist (44.6%). For detailed results please refer to Tables 2 and 3.

The responses on awareness of respondents towards consumer ADR center set up by the ministry of health seem to be quite interesting. More than half of the respondents (65.6%, \( n = 219 \)) reported unawareness about the existence of ADR center set up by the Ministry of Health. This showed statistical significance with respect to age (\( P = 0.039 \)).

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Majority of the respondents believed that the drugs marketed were not safe (81.1%, \( n = 271 \)). Among the respondents who felt that not all drugs that were marketed were safe, 52.4% were 18-25 years old and 39.8% hold bachelor degree. For detailed results refer to Table 4.

**Discussion**

This pilot study encompasses the knowledge and opinion toward ADR reporting and its system among the general public.
of Penang, Malaysia. An interview-administered questionnaire was used and this is considered to be one of the best modes to reach the general population. The response rate was found to be 90.7%. However, during the survey, there is no potential for external factors such as media reports or articles regarding ADR reporting to be consulted and would not supposedly wait for the respondents’ answers; therefore one can say that the results are valid and more or less reflect the understanding of the general population on ADR reporting.

In the current study, irrespective of their educational background, participants did not report any experience of side-effects due to their medications. Likewise, respondents in the senior age group did not report any side-effect from their medicines. This result seems to be very subjective and did not reflect real life situation. Generally, as the age advances, one can become more prone to develop side effects owing to decline in organ function. Moreover, this also highlights that participants might not have proper knowledge about the side-effects or either adverse effects of their prescribed medications. A study conducted in United Kingdom reported poor knowledge of the potential side-effects of their medications, but the patients were able to identify ADRs related to their medicines because of either unexpected nature of the symptom or timing. It is therefore, imperative on the part of provider, which could be either a doctor or a pharmacist to give adequate and sufficient information about their medications and to inform the patient about the reporting of any unexpected symptoms to their provider.

An interesting finding in the current study is the unawareness about consumer drug reaction center set up by the Ministry of Health. Although seems to be an expected finding, measures should be needed to well-inform the patients about how to report ADRs and committees to which these ADRs are reported. This result is in line with the previous studies in which the health-care professionals and the patients were not only unsure about the reporting, but did not have any prior information about the reporting centers in their regions.

Sources of information such as internet, newspapers, televisions, and mass media campaigns seem to play a key role in reporting ADRs. In the current study, majority of the respondents receive information about their drugs and side-effects from their doctors and pharmacists. Interestingly, advices from relatives and or friends were considered unprofessional in the current study and lesser taken into account. Quite a few of the respondents categorized television as their source of information. A study from France in 2002 reported that consumers were asked to make telephone calls for registering the side-effects to pharmaceutical companies and the companies entered these reports to drug safety database. Despite this, the personnel involved had divided opinions about the usefulness of this mode of reporting. On the contrary van van Hunsel et al. (2009) reported a peak in patient reporting of ADRs at Netherlands Pharmacovigilance Center Lareb between March 2007 and August 2007. The TV program is cited as a rational for drug discontinuation in nearly 50 reports. The study also highlighted that patients feel both lack of information and inadequate information from their health-care professionals.

In the current study, majority of the respondents reported that they will report to doctors about any side-effects while nearly a quarter of them reported that they will report to the pharmacists. There are studies, which reported that the detection of ADRs be improved provided the pharmacists be reported rather than medical doctors as pharmacists can improve ADR reporting. In a nutshell, patient reporting might have the potential to incorporate meaningful consequences to pharmacovigilance.

Conclusion

Respondents reflected inadequate knowledge on ADR reporting. This needs to be corrected as the trend of future pharmacovigilance is toward the patient. Moreover, the new trend seems to be more appropriate as the patient is the group of the people who are directly affected from the ADR of a particular drug and not the health-care providers. Therefore, it is essential for them to provide the sufficient knowledge toward reporting and reporting centers. Furthermore, specific area of the pharmacovigilance, which needs more attention includes patient understanding toward the basic concepts of ADR and how the patient will be benefited by ADR reporting and as well as what should be reported. Furthermore, the patient will be informed about the economic implications of not reporting ADR. It is recommended that government agencies, like MADRAC needs to find ways to increase patient-reported ADR cases.

The findings suggest that direct consumer education by health-care providers and the other relevant stakeholders’ bodies on issues pertaining ADRs detection and reporting could accelerate the involvement of consumers in ADRs reporting process.

Limitations of the study

The sample size is not reflective of general opinion of high educational background as we were unable to recruit respondents with a post-graduate degree.

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Anti-obesity activity of chloroform-methanol extract of *Premna integrifolia* in mice fed with cafeteria diet

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**ABSTRACT**

**Aim of the study:** Aim of the present study was to evaluate the anti-obesity activity of chloroform:methanol extract of *P. integrifolia* (CMPI) in mice fed with cafeteria diet. **Materials and Methods:** Female Swiss Albino mice were divided into six groups, which received normal and cafeteria diet, standard drug simvastatin (10 mg/kg) and CMPI (50, 100 and 200 mg/kg) daily for 40 days. Parameters such as body weight, body mass index (BMI), Lee index of obesity (LIO), food consumption, locomotor behavior, serum glucose, triglyceride, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL), atherogenic index, organ weight and organ fat pad weight were studied for evaluating the anti-obesity activity of *P. integrifolia*. High performance liquid chromatography (HPLC) fingerprint profile of chloroform-methanol extract was also studied using quercetin as the reference standard. **Results:** There was a significant increase in body weight, BMI, LIO, food consumption, organ weight (liver and small intestine), organ fat pad weight (mesenteric and peri-renal fat pad) and in the levels of serum glucose, triglyceride, total cholesterol, LDL and VLDL with a significant decrease in locomotor behavior (ambulation, rearing, grooming) and HDL level in cafeteria diet group. Animals treated with CMPI showed dose dependent activity. *P. integrifolia* (200 mg/kg) supplementation attenuated all the above alterations, which indicates the anti-obesity activity. HPLC fingerprint profile of CMPI showed two peaks in the solvent system of 50 mm potassium diphosphate (pH-3 with ortho phosphoric acid): Methanol (30:70 v/v) at 360 nm. **Conclusion:** Present findings suggest that, CMPI possessed anti-obesity activity that substantiated its ethno-medicinal use in the treatment of obesity.

**KEY WORDS:** Cafeteria diet, chloroform-methanol extract (1:1), obesity, *Premna integrifolia*

Introduction

Obesity is a serious illness that can lead to many medical complications. It results from an imbalance between food intake and energy expenditure, culminating in excessive accumulation of fat in adipose tissue, liver, muscle, pancreatic islets, and other organs involved in metabolism. Its prevalence is on a continuous rise in all age groups of many of the developed countries in the world. Obesity is rapidly turning into an epidemic afflicting much of the industrialized world, resulting in a prohibitive health and economic burden on society. Obesity is a multifactorial, chronic disorder that has reached a pandemic proportion world-wide. Nearly one third of the world’s adult population (1.3 billion people) was overweight or obese in 2005 and if recent trends continue, by 2030 nearly two third of the world’s adult population (3.3 billion people) could be either overweight or obese. Moreover, obese and overweight patients are at higher risk from coronary artery disease, hypertension, hyperlipidemia, diabetes mellitus, cancers, gall bladder disorders, cerebrovascular accidents, osteoarthritis, restrictive pulmonary disease and sleep apnea. Recently, much attention has been focused on some food factors/natural compounds that may be beneficial in preventing high fat diet induced body fat accumulation and possibly reduce the risk of diabetes and heart disease.

*P. integrifolia* Linn. (Verbenaceae), commonly known as Amni or Agnimantha[1,2] have been widely used for obesity and other obesity associated disorders. *P. integrifolia* has been reported for...
its potential actions such as antidiabetic and hypoglycemic, anti-inflammatory, immunomodulatory, cardiac stimulant, analgesic and antibacterial, anti-arthritic, antioxidant, hepatoprotective and in-vitro cytotoxic, antithyperglycaemic, antiparasitic, and hypolipidemic. The plant mainly contains p-methoxy cinnamic acid and linoleic acid, linoleic acid, β-sitosterol and flavone luteolin, iridoid glycoside, premnane, gianarine and ganikarine, prennazole, aphenol, pentacyclic terpene betulin, caropyphellon, premenol, prennaspirodiene, clerodendrin-A, three diterpenoids namely 1 β,3 α,8 β-trihydroxy-pimara-15-ene, 6 α,11,12,16-tetrahydroxy-7-oxo-abieta-8,11,13-triene and 2 α,19-dihydroxy-pimara-7,15-diene. Moreover, alkaloids, proteins, carbohydrates, amino acids, steroids, flavonoids, glycosides, tannins and phenolic compounds were found in preliminary phytochemical screening of CMPI. Therefore, the objective of the present study was to evaluate the anti-obesity activity of CMPI in mice fed with cafeteria diet in preclinical experimental animal model.

Materials and Methods

Chemicals and reagents

Glucose, triglyceride, total cholesterol, high density lipoprotein (HDL) kits were obtained from ERBA Diagnostics, Mannheim GmbH, Germany. Cafeteria diet was purchased from local market of Bhopal, Madhya Pradesh. Simvastatin was obtained from USV Ltd., Baddi, India. Petroleum ether, chloroform and methanol were obtained from SD Fine-Chem Ltd, Mumbai, India. All other chemicals and reagents used for experimental work were of analytical grade.

Plant material and preparation of extract

Fresh, well developed P. integrifolia plants and their roots were collected from the region of North Karnataka, India in the month of September 2008 and it was authenticated by a taxonomist, Department of Botany, Basaveshwar Science College, B.V.V.S. Campus, Bagalkot, Karnataka, India. Voucher specimen (No. B.Sc/Bot/13/08) was deposited in the College, B.V .V. S. Campus, Bagalkot, Karnataka, India. Inbreed animals were obtained from the central animal house of Radharaman College of Pharmacy, Bhopal. The animals were housed at room temperature (25 ± 1°C) with 50 ± 5% relative humidity and given standard laboratory feed (Hindustan Lever, India) and water ad libitum throughout the experimental period. The study was approved by Institutional Animal Ethics Committee, Radharaman College of Pharmacy, Bhopal.

Acute toxicity study

Acute toxicity study of CMPI was performed as per the Organization for Economic Co-operation and Development (OECD) guideline No. 425 and 420 followed by up and down and Fixed dose method. Based on these agreements, a limit test was performed to categorize the toxicity class of the compound and then main test was performed to estimate the exact 50% of lethal dose (LD₅₀). The dose range of 50, 100 and 200 mg/kg was selected for CMPI. The doses selected for the study starts from 1/10, 1/20 and 1/50 of the LD₅₀.

Experimental protocol for anti-obesity activity

Female Swiss albino mice (22-26 gm) were randomly divided into six groups of six mice in each and treated are as follows: Group I: Received standard laboratory feed, i.e., the normal diet. Group II: Received cafeteria diet in pellets forms. Group III: Simvastatin (10 mg/kg, orally) was administered daily. Group IV: CMPI (50 mg/kg, orally) was administered daily. Group V: CMPI (100 mg/kg, orally) was administered daily. Group VI: CMPI (200 mg/kg, orally) was administered daily.

Preparation of cafeteria diet for induction of obesity

The method described by Harris and Kulkarni was followed with some modifications. Cafeteria diet (highly palatable, energy rich animal diet that includes a variety of human snack foods) consists of 3 diets, which includes (condensed milk 48 g + bread 48 g), (chocolate 18 g + biscuits 36 g + dried coconut 36 g), (cheese 48 g + boiled potatoes 60 g). Cafeteria diet.
diet was presented in the form of pellets to 5 groups of 6 mice in each for 40 days.

**Parameters evaluated**

**Body weight**

Body weights of mice (g) were recorded on 1, 10, 20, 30 and 40 day in each group.[43]

**Body mass index and lee index of obesity**

Body mass index (BMI) and lee index of obesity (LIO) of mice were recorded on 1 and 40 day of study (i.e., initial and final body weight and body height) and was measured by using formulas:[45]

\[
\text{BMI} = \text{Body weight in gm}/(\text{Height in cm})^2
\]

\[
\text{LIO} = \text{Body weight in gm}/(1/3)/\text{Nasoanal length in cm}
\]

**Food consumption**

Food consumption study was carried out on 1, 10, 20, 30 and 40 day and recorded at 1 h, 2 h and 3 h of time intervals. The food consumption was estimated by subtracting the amount of food left on the grid from initial food weight.[46]

**Locomotor behavior**

Locomotor behavior study was performed on 40 day by using open field behavior test after 30 min of drug administration. Open field test was performed by placing mice in the center of apparatus and recording the ambulation (by counting the number of horizontal and vertical compartments traversed by animal), frequency of rearing and grooming for a 5 min test period was recorded.[43,47,48]

**Biochemical profile**

On 41 day, the blood samples were taken by penetrating the retro-orbital plexus with a fine glass capillary. The blood samples were centrifuged at 2500 rpm for 15 min to separate the serum and preserved (−20°C) for analysis of glucose (Trinder’s Method), triglycerides (Glycerol phosphate oxidase (GPO)-Trinder Method, End Point), total cholesterol (Modified Roeschau’s Method), high density lipoprotein-c (HDL-c) (Phosphotungstic Acid Method), low density lipoprotein-c (LDL-c) (Calculated using Friedewald’s equation, LDL-c = total cholesterol− very low density lipoprotein (VLDL-c – HDL-c), VLDL-c (VLDL-c = Triglyceride/5) and atherogenic index (AI = LDL-c + VLDL-c/HDL-c) were estimated.[43,49,50]

**Organ weight**

Mice were sacrificed under diethyl ether anesthesia on 41 day. Organs such as brain, liver, stomach, heart, small intestine, spleen, lungs, kidneys, and adrenal glands were removed and weighed.[41]

**Organ fat pad weight**

**Mesenteric fat pad**

Mice were sacrificed under diethyl ether anesthesia on 41 day. The fat deposited on mesentery, i.e., duodenum to the large intestine were removed and weighed.

**Peri-renal fat pad**

Mice were sacrificed under diethyl ether anesthesia on 41 day. The fat deposited on left and right kidneys were removed and weighed.[43]

**Statistical analysis**

All experimental data were statistically analyzed and expressed as means ± SEM. The significance of difference between control and treated animals for different parameters were determined by using one-way analysis of variance followed by multiple comparisons Dunnett’s test. \( P < 0.05 \) value was considered as statistically significant.

**Results**

**Effect of CMPI on body weight**

Table 1 shows the effect of CMPI on body weight in normal and experimental group of mice. Cafeteria diet group showed significant \( P < 0.001 \) increase in body weight on 10, 20, 30, and 40 day as compared to a normal diet group. Cafeteria diet fed mice treated with simvastatin showed significant \( P < 0.05-P < 0.001 \) decrease in body weight on 10, 20, 30 and 40 day as compared to cafeteria diet group. Oral

| Treatment groups          | 1 day          | 10 day         | 20 day         | 30 day         | 40 day         |
|---------------------------|----------------|----------------|----------------|----------------|----------------|
| Normal diet               | 23.01±1.78     | 23.32±1.83     | 23.99±1.86     | 24.52±1.93     | 25.32±2.14     |
| Cafeteria diet            | 24.54±1.94     | 27.67±2.55*    | 28.26±2.79*    | 30.83±2.98*    | 32.42±3.04*    |
| Cafeteria diet + simvastatin | 24.20±1.52   | 23.85±1.89*    | 21.25±1.28*    | 20.87±1.19*    | 19.80±1.05*    |
| Cafeteria diet + CMPI (50 mg/kg) | 24.12±1.48 | 26.78±2.36     | 26.03±2.28     | 25.80±2.17*    | 25.05±2.06*    |
| Cafeteria diet + CMPI (100 mg/kg) | 24.80±1.83 | 26.13±2.17     | 25.67±2.12     | 25.01±2.06*    | 24.61±1.96*    |
| Cafeteria diet + CMPI (200 mg/kg) | 24.88±1.89 | 25.49±2.10     | 24.73±1.77*    | 23.59±1.87*    | 21.73±1.33*    |

All values are expressed as a mean±SEM, \( n=6 \), CMPI: Chloroform-methanol extract of *Premna integrifolia*, \( ^*P<0.001 \) compared with normal diet group and \( ^{4}P<0.05, ^{4}P<0.01, ^{4}P<0.001 \) compared with cafeteria diet group.
administration of CMPI group (200 mg/kg) showed significant ($P < 0.05$-$P < 0.01$) reduction in body weight at 20, 30 and 40 day as compared to cafeteria diet group.

**Effect of CMPI on BMI and LIO**

Effect of CMPI on BMI and LIO in normal and experimental group of mice is shown in Table 2. Feeding the cafeteria diet to mice was found to significantly ($P < 0.001$) increase the final BMI and LIO when compared to mice fed with normal diet. Cafeteria diet fed mice treated with simvastatin, CMPI (200 mg/kg) groups showed a significant ($P < 0.001$) decrease in the final BMI and LIO when compared with cafeteria diet group.

**Effect of CMPI on food consumption**

Table 3 shows the effect of CMPI on food consumption in normal and experimental group of mice. Food consumption was found to be significantly ($P<0.001$) increased on 1, 10, 20, 30 and 40 day as compared to a normal diet group. Simvastatin administered mice showed a significant ($P < 0.01$, $P < 0.001$) decrease in food consumption on 30 and 40 day as compared to cafeteria diet group. The CMPI (200 mg/kg) treated mice showed significantly ($P < 0.05$, $P < 0.01$) decreased food consumption on 30 and 40 day as compared to cafeteria diet group.

**Effect of CMPI on locomotor behavior**

Table 4 shows the effect of CMPI on locomotor behavior in normal and experimental group of mice. Cafeteria diet group showed significant ($P<0.001$) decrease in ambulation, rearing and grooming as compared to a normal diet group. Cafeteria diet fed mice treated with simvastatin, CMPI (200 mg/kg) groups showed a significant increase in ambulation ($P<0.001$), rearing ($P<0.05$) and grooming ($P<0.05$) as compared to cafeteria diet group.

**Effect of CMPI on biochemical profile**

Effect of CMPI on biochemical profile in normal and experimental group of mice is shown in Table 5. Feeding the cafeteria diet to mice was found to significantly ($P < 0.001$) increase the levels of serum glucose, triglycerides, total cholesterol, LDL, VLDL, atherogenic index and significantly ($P < 0.001$) decrease the level of HDL when compared to mice fed with normal diet. Cafeteria

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**Table 2: Effect of CMPI on BMI and LIO in normal and experimental group of mice**

| Treatment groups       | Initial BMI (g/cm²) | Final BMI (g/cm²) | Initial LIO (g²/cm) | Final LIO (g²/cm) |
|------------------------|---------------------|------------------|---------------------|------------------|
| Normal diet            | 0.38±0.02           | 0.42±0.03        | 59.0±4.1            | 64.9±5.3         |
| Cafeteria diet         | 0.41±0.03           | 0.54±0.05        | 62.9±4.6            | 83.2±7.5         |
| Cafeteria diet + simvastatin | 0.40±0.03         | 0.33±0.02        | 61.9±4.3            | 50.8±3.2         |
| Cafeteria diet + CMPI (50 mg/kg) | 0.40±0.03       | 0.42±0.03        | 63.6±4.9            | 64.2±5.2         |
| Cafeteria diet + CMPI (100 mg/kg) | 0.41±0.03        | 0.41±0.03        | 63.6±4.9            | 63.1±4.9         |
| Cafeteria diet + CMPI (200 mg/kg) | 0.41±0.03        | 0.36±0.02        | 63.8±5.0            | 55.7±3.7         |

All values are expressed as a mean±SEM, n=6, CMPI: Chloroform-methanol extract of *Premna integrifolia* root, BMI: Body mass index, LIO: Lee index of obesity, $^A P<0.001$ compared with normal diet group and $^B P<0.05$, $^C P<0.01$, $^D P<0.001$ compared with cafeteria diet group.

**Table 3: Effect of CMPI on food consumption in normal and experimental group of mice**

| Treatment groups       | Days and food consumption (g) |
|------------------------|-------------------------------|
|                        | 1 day                         | 10 day                      | 20 day                      | 30 day                      | 40 day                      |
| Normal diet            | 2.74±0.15                     | 1.76±0.10                   | 3.12±0.21                   | 2.10±0.12                   | 3.38±0.29                   |
| Cafeteria diet         | 16.8±1.54                     | 13.5±1.23                   | 12.1±1.18                   | 11.2±1.03                   | 10.2±0.90                   |
| Cafeteria diet + simvastatin | 16.4±1.54                | 12.3±1.18                   | 10.3±0.92                   | 8.70±0.70                   | 5.40±0.52                   |
| Cafeteria diet + CMPI (50 mg/kg) | 17.1±1.60               | 13.4±1.23                   | 13.2±1.23                   | 11.9±1.06                   | 10.2±0.92                   |
| Cafeteria diet + CMPI (100 mg/kg) | 15.9±1.38              | 13.2±1.23                   | 13.3±1.23                   | 10.7±1.01                   | 8.10±0.63                   |
| Cafeteria diet + CMPI (200 mg/kg) | 16.7±1.58              | 12.9±1.21                   | 10.4±0.92                   | 9.70±0.80                   | 7.70±0.57                   |

All values are expressed as a mean±SEM, n=6, CMPI: Chloroform-methanol extract of *P. integrifolia* root, $^A P<0.001$ compared with normal diet group and $^B P<0.05$, $^C P<0.01$, $^D P<0.001$ compared with cafeteria diet group.

**Table 4: Effect of CMPI on locomotor behavior in normal and experimental group of mice**

| Treatment groups       | Ambulation | Rearing | Grooming |
|------------------------|------------|---------|----------|
| Normal diet            | 128.3±11.8 | 3.16±0.25 | 13.6±0.93 |
| Cafeteria diet         | 43.17±3.7² | 1.50±0.14³ | 6.66±0.57³ |
| Cafeteria diet + simvastatin | 110.5±10.4² | 2.70±0.22⁴ | 10.6±0.97⁴ |
| Cafeteria diet + CMPI (50 mg/kg) | 57.1±4.7²  | 1.83±0.15  | 8.5±0.77  |
| Cafeteria diet + CMPI (100 mg/kg) | 63.3±5.1²  | 1.96±0.17  | 8.1±0.74  |
| Cafeteria diet + CMPI (200 mg/kg) | 100.3±9.4² | 2.53±0.20⁴ | 9.8±0.86⁴ |

All values are expressed as a mean±SEM, n=6, CMPI: Chloroform-methanol extract of *Premna integrifolia*, $^A P<0.001$ compared with normal diet group and $^B P<0.05$, $^C P<0.01$, $^D P<0.001$ compared with cafeteria diet group.
diet fed mice treated with simvastatin, CMPI (200 mg/kg) groups showed a significant \( P < 0.001 \) decrease in the levels of serum glucose, triglycerides, total cholesterol, LDL, VLDL, AI and significant \( P < 0.001 \) increase in the levels of HDL when compared with cafeteria diet group.

### Effect of CMPI on organ weight

Table 6 shows the effect of CMPI on organ weight in normal and experimental group of mice. The weight of organs such as liver and small intestine in cafeteria diet group was found to be significantly \( P < 0.001 \) increased as compared to a normal diet group. Simvastatin administered mice showed a significant decrease in organ weight such as liver \( P < 0.01 \) and small intestine \( P < 0.001 \) as compared to cafeteria diet group. The CMPI (200 mg/kg) treated mice showed significantly \( P < 0.05 \) decreased weight of liver and small intestine as compared to cafeteria diet group. There was no significant difference between the organ weights of brain, stomach, heart, spleen, lungs, kidneys and adrenal glands of cafeteria diet group and other experimental groups.

### Effect of CMPI on organ fat pad weight

Effect of CMPI on organ fat pad weight in normal and experimental group of mice is shown in Table 7. Feeding the cafeteria diet to mice was found to significantly \( P < 0.01, P < 0.001 \) increase the weights of mesenteric and peri-renal fat pads when compared to mice fed with normal diet. Cafeteria diet fed mice treated with simvastatin, CMPI (200 mg/kg) groups showed a significant \( P < 0.01, P < 0.001 \) decrease in the weights of mesenteric and peri-renal fat pads compared with cafeteria diet group.

### Discussion

Obesity is arguably biggest challenge among the epidemics facing world-wide.\(^{\text{[51]}}\) A cafeteria diet induced obesity model is the simplest obesity induction model and possibly the one that most closely resembles the reality of obesity in humans.\(^{\text{[52]}}\) Cafeteria diet has been previously reported to increase energy intake and cause obesity in humans\(^{\text{[53]}}\) as well as in animals.\(^{\text{[54]}}\) The cafeteria diet has been reported to induce hyperphagia in rats,\(^{\text{[55]}}\) which results in higher fats stores resulting in increased body weight and organ weight.\(^{\text{[56]}}\) The results of our present study was in line with the above findings as we have observed a significant increase in body weight, BMI, LIO, food consumption, organ weight (liver and small intestine), and organ fat pad weight (mesenteric and peri-renal fat pad) in cafeteria diet induced group. The elevated consumption of foods rich in calories such as high fat food is associated with the low physical activity. Moreover, studies have shown that mice fed a high fat diet displayed lower frequencies of ambulation, rearing and grooming as compared to mice fed with a balanced diet.\(^{\text{[57,58]}}\) These results were in agreement with our present studies as we have noticed decreased ambulation, rearing and grooming in cafeteria diet group as compared to a normal diet group. Treatment with CMPI groups showed a significant increase in ambulation, rearing and grooming suggesting its beneficial role in maintaining the physical activity and locomotor behavior.

High fat diet induced obesity can lead to insulin resistance. Obesity is associated with a decreased capacity of insulin to regulate glucose metabolism in the peripheral tissues.\(^{\text{[59]}}\) Reports have strongly suggested that obesity is strong associated with imbalance in glucose and insulin homeostasis.\(^{\text{[60]}}\) In our present study, cafeteria diet fed mice showed abnormally increased blood glucose levels. Treatment with \( P. \ integrifolia \) markedly brought down the levels of blood glucose suggesting its anti-hyperglycemic effect. Obesity is associated with an unfavorable lipid profile. Lipid abnormalities related to obesity include an elevated serum concentration of triglycerides, cholesterol, LDL and VLDL as well as a reduction in serum HDL.\(^{\text{[61]}}\) It is shown that cafeteria diet elevates serum triglyceride levels essentially by preventing its uptake and clearance by inhibiting catabolizing enzymes like lipoprotein lipase (LPL) and lecithin cholesterol acetyl transferase.\(^{\text{[62]}}\) Cafeteria diet induced hypercholesterolemia has been related to its ability to alter the physico-chemical properties of lipoproteins and thereby prevent their uptake by the liver for clearance.\(^{\text{[63]}}\) High fat diet increases both LDL-cholesterol and oxidative stress that results in increased oxidized LDL levels leading to atherosclerotic plaque formation.\(^{\text{[64]}}\) Moreover, cafeteria diet decreases the levels of HDL, which is considered to be the good cholesterol that is anti-atherogenic in nature.\(^{\text{[65]}}\) The above findings were in supportive with our present results as we have observed increased serum levels of triglycerides, total cholesterol, LDL, VLDL, and decreased levels of serum HDL in cafeteria diet induced animals. Animals treated with \( P. \ integrifolia \) showed markedly reduced serum levels of triglycerides, total cholesterol, LDL, VLDL, and increased levels of serum HDL. The reduction in triglycerides

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| Treatment groups           | Glucose (mg/dl) | Triglyceride (mg/dl) | Total cholesterol (mg/dl) | HDL (mg/dl) | LDL (mg/dl) | VLDL (mg/dl) | AI (Units) |
|----------------------------|-----------------|----------------------|---------------------------|-------------|-------------|--------------|------------|
| Normal diet                | 74.5±6.3        | 66.5±6.2             | 162.5±15.4                | 37.5±3.1    | 111.6±10.5  | 13.3±1.2     | 3.33±0.3   |
| Cafeteria diet             | 94.7±8.7\(^{\text{a}}\) | 99.2±9.5\(^{\text{c}}\) | 194.2±18.6\(^{\text{c}}\) | 27.6±2.9\(^{\text{c}}\) | 147.0±13.5\(^{\text{c}}\) | 19.8±2.1\(^{\text{c}}\) | 6.04±0.5\(^{\text{c}}\) |
| Cafeteria diet+simvastatin | 75.9±6.1\(^{\text{c}}\) | 70.3±6.8\(^{\text{c}}\) | 172.8±16.3\(^{\text{c}}\) | 35.3±2.9\(^{\text{c}}\) | 123.3±11.6\(^{\text{c}}\) | 14.2±1.3\(^{\text{c}}\) | 3.89±0.4\(^{\text{c}}\) |
| Cafeteria diet+CMPI (50 mg/kg) | 82.2±7.8\(^{\text{c}}\) | 85.6±8.1\(^{\text{c}}\) | 187.6±18.2\(^{\text{c}}\) | 29.4±2.9\(^{\text{c}}\) | 141.1±12.7\(^{\text{c}}\) | 17.1±1.9\(^{\text{c}}\) | 5.38±0.4\(^{\text{c}}\) |
| Cafeteria diet+CMPI (100 mg/kg) | 82.9±7.8\(^{\text{c}}\) | 82.1±7.7\(^{\text{c}}\) | 184.9±17.7\(^{\text{c}}\) | 32.7±2.7\(^{\text{c}}\) | 135.8±12.1\(^{\text{c}}\) | 16.4±1.7\(^{\text{c}}\) | 4.65±0.3\(^{\text{c}}\) |
| Cafeteria diet+CMPI (200 mg/kg) | 76.8±6.2\(^{\text{c}}\) | 74.5±7.1\(^{\text{c}}\) | 177.7±17.2\(^{\text{c}}\) | 34.3±2.8\(^{\text{c}}\) | 128.4±11.8\(^{\text{c}}\) | 15.0±1.5\(^{\text{c}}\) | 4.18±0.3\(^{\text{c}}\) |

All values are expressed as a mean±SEM, \( n = 6 \), CMPI: Chloroform-methanol extract of \( P. \ integrifolia \), HDL: High density lipoprotein, LDL: Low density lipoprotein, VLDL: Very low density lipoprotein, AI: Atherogenic index, \( ^{\text{a}}P<0.001 \) compared with normal diet group and \( ^{\text{b}}P<0.05, ^{\text{c}}P<0.01 \), \( ^{\text{c}}P<0.001 \) compared with cafeteria diet group.

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Table 6: Effect of CMPI on organ weight in normal and experimental group of mice

| Treatment                        | Brain (g) | Liver (g) | Stomach (g) | Heart (g) | Small intestine (g) | Spleen (g) | Lungs (g) | Kidneys (g) | Adrenal glands (g) | Right | Left | Right | Left | Right | Left |
|----------------------------------|-----------|-----------|-------------|-----------|--------------------|------------|-----------|-------------|-------------------|-------|------|-------|------|-------|------|
| Normal diet                      | 0.28±0.02 | 0.99±0.06 | 0.28±0.02   | 0.09±0.005| 0.92±0.05          | 0.11±0.001 | 0.17±0.001| 0.12±0.001   | 0.11±0.001         | 0.02±0.02 | 0.11±0.002 | 0.02±0.02 | 0.02±0.02 | 0.02±0.02 | 0.02±0.02 |
| Cafeteria diet                   | 0.29±0.02 | 1.18±0.07 | 0.35±0.03   | 0.16±0.009| 1.56±0.17          | 0.15±0.11  | 0.12±0.01  | 0.12±0.11    | 0.12±0.01          | 0.02±0.02 | 0.16±0.004 | 0.02±0.02 | 0.02±0.02 | 0.02±0.02 | 0.02±0.02 |
| Cafeteria diet+simvastatin       |          |          |             |           |                    |            |           |             |                   |        |      |       |      |       |      |
| Cafeteria diet+CMPI (50 mg/kg)   |          |          |             |           |                    |            |           |             |                   |        |      |       |      |       |      |
| Cafeteria diet+CMPI (100 mg/kg)  |          |          |             |           |                    |            |           |             |                   |        |      |       |      |       |      |
| Cafeteria diet+CMPI (200 mg/kg)  |          |          |             |           |                    |            |           |             |                   |        |      |       |      |       |      |

All values are expressed as a mean±SEM, n=6, CMPI: Chloroform-methanol extract of *Premna integrifolia*.

The decline in VLDL levels in treated groups could be directly correlated to a decline in triglyceride levels as it is well established that VLDL particles are the main transporters of triglycerides in plasma. A decrease in serum cholesterol and LDL level is due to decreased cholesterol absorption from the intestine by binding with bile acids within the intestine and increasing fecal bile acids excretion. Tannins present in *P. integrifolia* have been reported to increase fecal bile acid excretion, thereby leading to reduction in cholesterol levels. Elevated levels of HDL helps to carry cholesterol back to the liver, where it gets excreted as bile salts. In our present study, mice fed with *P. integrifolia* were found to significantly increase serum HDL levels suggesting its cardioprotective nature. The increased level of HDL observed in CMPI compare to the cafeteria diet group may be due to an increased activity of lecithin cholesterol acyl transferase (an enzyme which incorporates free cholesterol from LDL into HDL and transfer it back to VLDL and intermediate density lipoprotein).
Table 7: Effect of CMPI on organ fat pad weight in normal and experimental group of mice

| Treatment groups | Mesenteric fat pad (g) | Peri-renal fat pad (g) |
|------------------|------------------------|------------------------|
| Normal diet      | 0.13±0.003             | 0.011±0.002            |
| Cafeteria diet   | 0.38±0.009             | 0.015±0.003            |
| Cafeteria diet+simvastatin | 0.15±0.004²         | 0.012±0.002²           |
| Cafeteria diet+CMPI (50 mg/kg) | 0.25±0.007⁴        | 0.013±0.002            |
| Cafeteria diet+CMPI (100 mg/kg) | 0.22±0.007⁴         | 0.014±0.002            |
| Cafeteria diet+CMPI (200 mg/kg) | 0.19±0.006²         | 0.013±0.002²           |

All values are expressed as a mean±SEM, n=6, CMPI: Chloroform-methanol extract of Premna integrifolia, ²P<0.05, ³P<0.01, ⁴P<0.001 compared with normal diet group and ²P<0.05, ³P<0.01, ⁴P<0.001 compared with cafeteria diet group.

index of plasma has recently been proposed as a marker of plasma atherogenicity because it is increased in people at higher risk for coronary heart diseases and is inversely correlated with LDL particle size.⁷⁰,⁷¹ In our present study, treatment with P. integrifolia showed a reduction in atherogenic index, which could be due to the presence of alkaloids (premnazole), flavonoids (luteolin), sterols (β-sitosterol), amino acids (linoleic acid-fatty acid), carbohydrates, tannins and polyphenolic compounds.¹⁴,²²,³⁴-⁴⁰ However, HPLC fingerprint profile of CMPI [Figure 1] showed two peaks in the solvent system of 50 Mn potassium phosphate (pH-3 with Ortho Phosphoric acid): Methanol (30:70 v/v) at 360 nm. The reference standard was used as quercetin [Figure 2]. Thus, it is suggested that assessment of active constituents and clinical evaluation of P. integrifolia would give a positive lead in the successful treatment of obesity. However, further series of studies are required to prove its clinical reliability, safety and efficacy.

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Epilepsy is the most common chronic neurological disorder in humans. The first report of gingival enlargement associated with the chronic use of phenytoin was made in 1939. This agent remains as one of the most commonly prescribed medications to treat epilepsy and it may also be used in cases of neuralgias and cardiac arrhythmias. It is estimated that about 30-50% of patients taking phenytoin develop significant gingival alterations. Not all patients taking phenytoin develop phenytoin induced gingival enlargement. Evidence suggests that genetic factors also might have a significant role in the pathogenesis of phenytoin induced gingival enlargement and in the patient’s susceptibility to this unwanted effect. A genetic predisposition could influence a variety of factors in the drug plaque-induced inflammation. These include gingival fibroblast functional heterogeneity, collagenolytic activity, drug metabolism, and collagen synthesis.

Regarding drug metabolism, the cytochrome P450 2C9 (CYP2C9) is responsible for phenytoin conversion on its hydroxylated form in the liver. Pharmacogenetic influences on drug metabolism have been widely reviewed and gene polymorphism of cytochrome P450 2C9 appeared to be responsible for much of the interindividual variability on drug elimination. Genetic variation in the CYP2C9 gene can affect metabolism, leading to altered phenotypes. Individuals with poor metaboliser alleles of CYP2C9 gene were shown to have a reduced metabolism of phenytoin compared with wild-type alleles. Thus identification of patients genotype prior to anti-epileptic drug administration could potentially prevent higher serum drug concentrations leading to adverse side effects such as gingival enlargement. This case report addresses the influence of CYP2C9 genetic polymorphism on Phenytoin drug metabolism thereby causing gingival enlargement.

Case Report

A 18-year-old male patient came to Department of Periodontics, Mahatma Gandhi Post Graduate Institute of Dental Sciences, Pondicherry, with a complain of swelling of upper and lower teeth gums for the past 10 months. Patient’s medical history revealed
that he had been on long-term phenytoin therapy (10 years) for primary generalized epilepsy at a daily dose of 300-mg/day. On intraoral clinical examination, there was generalized gingival enlargement involving marginal, attached, interdental papilla of upper and lower anterior teeth both labial and lingual/palatal aspects [Figures 1-3] and on the buccal aspects of upper and lower premolars and first and second molars. The enlargement was pale pink in color, firm, resilient with minutely lobulated surface and no tendency to bleed on probing. There was also presence of supra and subgingival deposits. There were no any other significant dental findings. Based on these clinical findings a provisional diagnosis of Phenytoin induced gingival enlargement with superimposed inflammatory component was made.

Routine blood investigations were done which were found to be within normal limits. Gingival enlargement was due to phenytoin toxicity. This phenytoin toxicity prompted appropriate pharmacogenomics studies. Patient was informed verbally and written informed consent was obtained. 5 ml of venous blood was collected from the antecubital vein into ethylenediaminetetraacetic acid tubes. Plasma was separated by centrifugation and stored at −20°C until the assay. After separation of plasma, the cellular fraction was subjected to DNA extraction from leukocytes by the standard phenol: Chloroform procedure and genotyping of CYP2C9 was performed by a polymerase chain reaction-restriction fragment length polymorphism method. The CYP2C9FNx012 (Arg144 Cys) was detected using the forward and reverse primers 5´ TACAAATACAATGAAAATATCATG 3´ and 5´ CTAACAACCAGACTCTATAATG 3´, respectively and the genotype result was found to be homozygous CYP2C9FNx013FNx013.

Discussion

Phenytoin is one of the most-widely prescribed anti-epileptic agents in the Indian population. This, combined with the fact that the CYP2C9 enzyme which metabolizes phenytoin is polymorphically expressed and that phenytoin has a narrow safety margin makes it important to study the effect of the CYP2C9 polymorphism with regard to phenytoin metabolism. Phenytoin has been found to be safe and suitable for use as a probe to study the pharmacogenetics of CYP2C9.[5] Phenytoin follows non-linear pharmacokinetics. At a concentration of <10 μg/ml, it follows first order (linear) kinetics, and beyond this, zero-order kinetics. Since the influence of enzymes can only be studied on the drugs that follow first-order kinetics, only a single dose of phenytoin was administered to the subjects, although epileptic patients receive long-term therapy in the clinical setting.

This 18-year-old male patient came with the complain of swelling in the gums, but prior to the treatment we planned for the pharmacogenomics study which proved to be homozygous mutant of CYP2C9 in our patient, but still larger samples should be taken to confirm the study. After the pharmacogenomic study, immediately drug substitution was done to sodium valproate under the patient’s physician opinion. Oral prophylaxis was done for the patient and was educated about the oral hygiene maintenance. After 6 months of drug substitution and oral hygiene maintenance the gingival enlargement was persisting in relation to upper and lower anteriors only and
hence Gingivectomy was planned for the patient and the excised tissue was sent for biopsy. The histopathological examination [Figure 4] revealed epithelial acanthosis, and elongated rete ridges. Hyperplasia of connective tissue, along with dense fibrous connective tissue was seen and there was a mild to moderate inflammatory cell infiltrate within connective tissue. Patient was recalled after 3 months, which revealed no evidence of recurrence [Figure 5].

**Conclusion**

Pharmacogenomics studies before initiating antiepileptic therapy could have easily identified the unfavorable genotype of CYP2C9, a major phenytoin metabolizing enzyme in this case, and prompted the physician to choose the correct dosage or to convince the patient on the need for an alternate antiepileptic drug such as sodium valproate. This would have avoided a number of iatrogenic complications of antiepileptic drug therapy. We recommend that wherever possible, the clinicians may do CYP2C9 genotyping of the epileptic patients before prescribing Phenytoin, with advancement in the molecular technology, the clinicians may do the genotypic analysis which enables them to have personalized prescription and to avoid the adverse drug reaction.

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Submandibular sialolithiasis: Report of six cases

Sir,

Sialolithiasis is considered to be the most common salivary gland disorder and it accounts for about 1.2% of unilateral major salivary gland swelling. Submandibular gland has got highest predilection for sialolithiasis with 80% occurrence rate, followed-by 19% in the parotid and 1% in the sublingual glands. Sialolithiasis usually appears between the age of 30 and 60 years, and it is uncommon in children as only 3% of all sialolithiasis cases has been reported in the pediatric population until to date. Males are affected twice as much as females.

Six cases of sialolithiasis with varying presentations and clinical features reported to our Departments of Oral Medicine and Radiology and Oral and Maxillofacial surgery in PSM College of Dental Sciences and Research. A brief discussion based on the clinical examination, investigation, and management of sialolithiasis is being reported.

A 55-year-old female patient came with a complaint of pain in the right mandibular region for 3 months. Previous history revealed a swelling on the same side 7 years back, which was then diagnosed as osteomyelitis and no treatment was carried out. Clinical examination revealed a swelling in the floor of the mouth on the right side with complete destruction of alveolar bone. Orocutaneous fistula with the myiasis was evident. Occlusal, computed tomography (CT) and Ortopantomograph confirmed the presence of sialolith. A diagnosis of osteomyelitis and sialolithiasis was given on the basis of clinical and the radiological examination [Figure 1].

A 25-year-old male patient came with a complaint of pain in the lower left back tooth region for a period of 5 days. Pain was recurrent in nature. There was no history of any increase of the swelling during meals. Clinical examination revealed an impacted 38 associated with a firm, tender swelling on the left floor of the mouth. Occlusal radiographic examination revealed two tubular radiopacities in the left side. A CT was taken, which confirmed the presence of calculi. A diagnosis of sialolith was given. Patient was posted for the surgical removal. Prior to surgery a clinical examination revealed a reduction of signs and symptoms. Repeat radiographs and CT revealed the absence of radiopacities. This led to a conclusion of spontaneous extrusion of sialolith [Figure 2].

Three cases of female patients also reported with the complaints of swelling in the floor of the mouth with pus discharge. Occlusal
Radiographic examination revealed radiopacities, which led to diagnosis of sialadenitis associated with the salivary calculi in all the three cases. Surgery was planned. Under antibiotic coverage, duct was isolated with an IV cannula, incision was placed longitudinally along the margins, and stone was identified. Calculi were removed in total and ductal walls were marsupilised. Healing was satisfactory in all the three cases [Figures 3-5].

An 8-year-old female patient reported with a swelling below the tongue, which was associated with pain for 1 week. Clinical examination revealed a superficial, 5 mm hard swelling situated near the lingual frenum, which was extremely tender on palpation. There was no associated discharge or bleeding reported from the area. Under local anesthetic, incision was placed at the ductal orifice and calculi was exposed and retrieved [Figure 6].

Regarding the occurrence of sialolith, in our series, all the cases were reported in the submandibular gland, which was consistent with the fact that it commonly occurs in the submandibular gland. The cases reported here were unusual, because the data didn't coincide with those reported in literature about the male predilection and rarity of occurrence in pediatric patients. A total of 90% of our patients were females, and one of our reported cases was of an 8-year-old child.

Acute sialadenitis of the submandibular gland is the most common form of inflammation to the major salivary glands. Obstruction of the salivary duct due to sialolithiasis is the most frequent etiology of sialadenitis. Hence patients with sialolithiasis can have acute pain due to sialadenitis and this was consistent with most of our cases.

Sialolithiasis is easy to diagnose on the basis of its clinical features. Occlusal radiographs are extremely useful in showing radiopaque stones unless otherwise there are radiolucent stones. Sialography is useful in patients showing signs of sialadenitis related to the radiolucent stones or deep submandibular/parotid stones. Sialography is contraindicated in acute infection or patients who are having contrast allergy. Ultrasonography with a 99% accuracy is considered as a gold standard in diagnosis. Newer techniques such as scintigraphy, CT, and sialoendoscopy has revolutionized the diagnostic aspect of sialolithiasis.

On the basis of a review of the literature, most of the sialolith are usually of 5 mm in maximum diameter and all the stones over 10 mm should be reported as a sialolith of unusual size. Some of our cases were more than 10 mm in size and can be considered as giant sialolith.

The management of sialolith is based on its location and the symptoms associated with it. Moist warm heat application with administration of sialogogues and gland massage helps in flushing the stone out of the duct.

Most stones will respond to antibiotics, combined with simple sialolithotomy. Under regional anesthesia, once the sialolith had been located, the orifice of the salivary duct has to be surgically enlarged with a long incision. A small pressure
exerted at the level of the distal ligature will provoke the discharge of the sialolith through the incision. Risks of this procedure include infection and bleeding. More uniquely to this procedure is the risk of duct scarring resulting in recurrent gland swelling. There is also a small risk of numbness to the floor of mouth region. In cases of bigger stones, prior fragmentation is necessary using an external lithotriptor or laser can be used if necessary. Stenoses in the main duct are treated with metallic dilators although, balloon catheters under endoscopic control is preferred when strictures are localized or situated in peripheral areas.[8]

Our case reports discuss sialolith presented in different sites such as submandibular gland and lingual frenum; its occurrence in a pediatric patient, and self‑extrusion of a sialolith. Diagnosis of salivary calculi is mainly based on clinical symptoms and imaging. Management is by surgical means and has to be performed only after managing any infection or inflammation. Even though, surgical treatment is the mainstay treatment modality at present, minimally invasive techniques like lithotrips will gain attention in the future.

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A medico-legal case of irresponsiveness to antibiotic therapy: A point that should not be forgotten

Sir,

The unresponsiveness to an appropriate medical treatment is an important concern in medical practice. There are many possible causes including, drug resistance, poor patient compliance, and poor quality of the drug (expiration, not pass quality control process, etc.). Apart from the mentioned common causes, there are also other causes to be addressed. Here, the author would like to present an experience on a case with the problem on unresponsiveness to standard antibiotic therapy. The indexed case was a 64-year-old female patient with the acute pyelonephritis and prescribed for the standard intravenous Cef-3 therapy. However, after 2 consecutive drug administrations, there is no improvement. The case was consulted for finding of the possible explanation. Of interest, after history taking, an interesting detail was derived. The patient described for the shape of the used drug ampule for her intravenous infusion and the detail seems not to be the shape of Cef-3. Further root cause investigation, shows that the in charge pharmacist get the order of the correct Cef-3 request in prescription form, but intend to give the vitamin B complex for injection. For sure, the disguised order was made and passed to the nurse in charge at drug administration room and the nurse in charge got that disguised order. This case was further managed as a possible corruption case and under legal management. Indeed, there are many possible unexpected causes of failure of drug treatment. Corruption can be seen in the underdeveloped country.1 The present medico-legal case can be the good case study to warn all physicians to concern on the problem that can occur in any steps on the way from drug prescription to administration to the patient. Based on this case, these recommendations are made:

1. There must be the repeatedly re-checking for the medical prescription. The final confirmation between nurse who perform drug administrator and physician in charge must be carried out in all cases.
2. The drug delivery system should be in the closed loop. The drug should be sent directly from the pharmacist to the nurse in-charge. The final reconfirmation with the physician in charge must be carried out.
3. There must be the routine auditing of the whole pathway. This is useful to detect the error as well as intentional corruption.

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