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In vivo cytotoxic effects of methanol extract of Convolvulus arvensis on 7-12-dimethyl benz(a)antheracene (DMBA) induced skin carcinogenesis

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A current study was conducted to evaluate the possible cytotoxic effect of Asian herb Convolvulus arvensis (methanolic extract), followed by 2 stage skin carcinogenesis protocol by tumor initiator, 7-12-dimethyl benz(a)antheracene (DMBA) and tumor promoter, Croton oil in Swiss albino mice. They induced 100% skin ulceration in carcinogen control and cumulative number of papilloma (CP), tumor yield (TY) and tumor burden (TB) were calculated as 18.20 ± 1.643, 3.640 ± 0.3286 and 3.640 ± 0.3286, respectively. Local application of the extract at 300 mg/kg/day inhibited the tumor incidence up to 20% in 16 weeks and showed a significant decline in continuous group in CP 4.800 ± 6.611 and TY 0.9600 ± 1.322 compared to carcinogen group. For assistance of morphological alteration, biochemical investigations were performed. Extract increased the reduced glutathione (GSH) from 3.286 ± 0.207 to 7.1260 ± 0.4953 μmol/g, superoxide dismutase (SOD) 1.722 ± 0.1262 to 6.5160 ± 0.3710 μmol/g and catalase (CAT) 13.624 ± 0.813 to 18.792 ± 0.714 of H₂O₂ reduction/mg protein/min and decreased lipid peroxidation (LPO) 7.652 ± 0.1863 to 4.2340 ± 0.5928 nmol/mg levels compared to carcinogen group. Histopathological changes showed papilomatosis and ulceration in carcinogen while acanthosis with normal psychological features in the continuous group. In conclusion, cytotoxic potential of Convolvulus arvensis methanol extract is due to the presence of querecitin, that is, flavonoid that have the ability to capture the reactive oxygen species, over expressions of tumor causing genes and topoisomerase II; its presence was confirmed by high performance of liquid chromatography (HPLC) chromatogram. These investigations may have introduced new phytochemicals that are easily available and effective for cancer prevention.

Key words: Convolvulus arvensis, skin carcinogenesis, tumor, 7-12-dimethyl Benz (a) antheracene (DMBA), croton oil, querecitin.

INTRODUCTION

Skin cancer is one of the most widespread diseases, characterized by malignancies on the outermost layer of
skin and related to the race, gender, age, geography (Marks, 2007). Sun rays, artificial light sources, chemicals and polycyclic aromatic hydrocarbons become the cause of carcinogenesis (Narayanan et al., 2010) which initiates by promotion and progression, and after metabolism, radicals of reactive oxygen species (ROS) are produced which mutate the properties, functions and sequence of amino acids (Dixon and Kopras, 2008; Park, 2005). In the present study an attempt has been made to study the cytotoxic activity of bindweed (Convolvulus arvensis) on the skin carcinogenesis induced by 7-12-dimethyl Benz (a) antheracene (DMBA) and croton oil in Swiss albino mice.

Convolvulus arvensis from family Convulvulaceae is commonly known as Morning-glory or bindweed widely found in Asia. Its aerial parts have flavonoids, tannins, polyphenols, caffeic acid, saponins, δ-amino levulinic acid, lipids and tropane alkaloids (Kaur and Kalia, 2012; Montes-Holguin et al., 2006) including cuscohygrine and calystegines (Atta and Mounier, 2004) and its leaves also contain various flavanoids and glycosides like Quercetin, Kaempherol-3-mono glycosides, etc. (Yusuf et al., 2002). Traditionally, the plant has been used for the treatment of fever, inflammation, hemorrhage, abdominal pain and worms in children (Atta and Mounier, 2004; Al-Bowait et al., 2010), skin ulcers and wound healing (Meng et al., 2002) flowers have laxative activity and useful for muscle weakness and leaves are immunostimulant and effective in asthma and jaundice (Al-Bowait et al., 2010). It has anti-angiogenesis and antioxidant activity at high doses due to the presence of proteoglycans and glycoprotiens (Meng et al., 2002; Calvino, 2002). Aerial parts have shown antioxidant and cytotoxic activity against Hela cells due to flavonoids (quercetin) and tannins (Sadeghi-alibadi et al., 2008, 2010). As we know that conventional medicines are in easy access, but due to their solemn side effects and high cost, phytotherapy are grabbing high interest (Surh, 2003). In ages proved antioxidant and free radical scavenging potential due to polyphenols, flavonoids, lignins, carotene, polysaccharides and xanthines like other anticancer agents vincristene, vinblastine, vincarosa, we had selected whole plant of Convolvulus arvensis for determining its in vivo cytotoxic potential.

MATERIALS AND METHODS

Collection of plant

Whole plants of Convolvulus arvensis were collected during the months of April and May in 35 to 40°C from the backyard of Govt. College, University Faisalabad, Pakistan and identified by a plant taxonomist Dr. Mansoor Hameed Head of Botany department, Agriculture University Faisalabad.

Preparation of plant extract

The whole plants were washed, chopped and dried under shade at room temperature for many days until fully dried, ground by electric grinder, powdered and sieved. This material was macerated in methanol for 7 days with frequent shaking every day, filtered out by using Whatman filter paper, separating out solvent from solid material by using a rotary evaporator at 40 to 50°C and residues obtained were stored in small amber jars at 4°C.

Drug or Chemical

Carcinogen 7-12-dimethyl benz(a)anthracene (DMBA) and Croton oil were obtained from Sigma Aldrich Chemical Company USA. Acetone was used as a vehicle for all topically applied carcinogens and dilution of plant extract.

Experimental animals

6 to 8 weeks old male mice weighing 20 to 30 g were obtained from National Institute of Health Islamabad and kept in animal house of the Department of Pharmacology, Government College, University Faisalabad under controlled conditions of Temperature (25 ± 1°C) and humidity (50 ± 5°C). They were given standard diet and water ad libitum. Mice were acclimatized to environment for one week prior to commencement of the experiment (Rosilda et al., 2011).

Experimental plan

Dorsal skin of albino mice was shaved with an electric clipper for approximately 2 x 2 cm area and marked with permanent marker (Arya and Kumar, 2011). 50 animals were divided into 5 subgroups: (1) Carcinogen Control group: 10 mice were applied topically with a single dose of DMBA as a tumor initiator on the shaved area of the skin of mice and two weeks later Croton oil was applied as tumor promoter thrice a week till the end of 16th week. (2) Pre group: 10 mice received 300 mg/kg methanol (CA) extract topically for consecutive 7 days. DMBA single topical dose was applied at 8th day and two weeks later Croton oil was applied thrice a week till the end of 16th week. (3) Peri group: 10 mice received DMBA single topical dose, then 300 mg/kg CA extract topically for consecutive 15 days. After that, Croton oil was applied thrice a week till the end of 16th week. (4) Post group: 10 mice were received DMBA single topical dose. After 2 weeks, 300 mg/kg CA extract applied topically daily with the half hour delay application of Croton oil thrice a week till the end of 16th week. (5) Continuous group: 10 mice were received 300 mg/kg C.A (methanol) extract topically throughout the experimental period daily and at 7th day DMBA single topical dose was applied and two weeks later Croton oil was applied thrice a week till the end of 16th week.

Preparation of stock solution

1 molar 100 μg DMBA was dissolved in 100 μl acetone and Croton oil was applied thrice a week till the end of 16th week.
oil at 1 μg/100 μl to make 1% (w/v) dilution prepared just before its use and kept in amber glass bottle at about 20°C. A stock solution of extract was prepared by dissolving 10 mg extract in 1.0 ml acetone. Serial dilution of 300 mg/kg was made (Rosilda et al., 2011).

**Preliminary phytochemistry**

Determining the chemical constituents in CA extract was done by following protocols.

**Screening for reducing sugars**

In determining the presence of reducing sugars, weighed 0.5 g of ethanolic extract was used to form aqueous ethanolic extract in 5 ml distilled water in a test tube and few drops of both Fehling’s solution A and B added. The change in color was observed. Color alterations showed the presence of reducing sugars (Khan et al., 2011).

**Screening for alkaloids**

For determining the alkaloids, to weighed 0.2 g of alcoholic extract was added 5 ml hydrochloric acid 2 N solution which was warmed in a boiling water bath, cooled and filtered properly. The obtained filtered solution was put in equal portions in two test tubes to which was added 2 to 3 drops of Mayer’s reagent in one tube and Dragendoff’s in the other. Suspending white creamy solid in Mayer’s reagent and reddish brown in Dragendoff’s showed the confirmation for the existence of alkaloids (Khan et al., 2011).

**Screening for tannins**

In determining the tannins presence, the weighed 0.5 g plant extract was dissolved in 10 ml distilled water, warmed till boiling then filtered properly. 0.1% solution of ferric chloride was added to infiltrate solution. Changed color from dark brown green or blue green showed the existence of tannins (Egwaikhide et al., 2007).

**Screening for saponins**

In determining the saponins presence, a weighed 0.5 g extract was put in the test tubes and to it was added 5 ml distilled water. This mixture was shaken and waited for visible foam to appear. Then a few drops of olive oil was added in this stable foam and an emulsion was formed which showed the presence of saponins (Khan et al., 2011).

**Screening for terpenoids**

In determining the presence of terpenoids, a weighed 0.2 g extract was mixed with 2 ml of chloroform and then sulfuric acid was added in small parts within this solution. A fine thin layer was formed and inner solution showing reddish brown color indicated the presence of terpenoids (Mojab et al., 2003).

**Screening for flavonoids**

In determining the presence of flavonoids, to a weighed 0.2 g extract, 5 ml of diluted sodium hydroxide and 5 ml of 1 M hydrochloric acid was added in plant extract. Yellowish red solution was formed that turned into a transparent white solution which indicated the existence of flavonoids (Mojab et al., 2003).

**Screening for anthraquinones**

In determining the presence of anthraquinones, a weighed 0.5 g plant extract was warmed for 3, 4 min with 10% hydrochloric acid in a boiling water bath then filtered properly and cooled for a few minutes. Methyl chloride in equal volume of filtrate was added in this solution. 10% ammonia was mixed with this solution before heating. A fine pink or dark pink color indicated the existence of anthraquinones (Egwaikhide et al., 2007).

**Screening for glycosides**

In determining the presence of glycosides, a weighed 1.2 g of plant extract was mixed in 10 ml of 1% hydro alcoholic acid and 10% sodium hydroxide and then to it was added 3 to 4 drops of both Fehling’s solutions A and B. Reddish precipitates indicated the existence of glycosides (Mojab et al., 2003).

**Determining the pharmacological activity**

Screening was done by the following studies.

**Morphological studies**

Skin of each mice was weekly observed for loss of hair, redness, ulceration and outgrowth. These were counted and measured by digital vernier caliper till the end of 16th weeks. Recorded morphological parameters were

1. Tumor incidence: Number of tumor bearing mice,
2. Cumulative number of papilloma, tumor yield: Average number of tumors per mouse and,
3. Tumor Burden: Number of tumors per tumor bearing mice (Rosilda et al., 2011).

**Biochemical studies**

Mice ulcerated skin was shaved, washed with cool and normal saline solution. Excised skin was used to prepare 10% tissue homogenate in 0.15 molar Tris potassium chloride having a pH of 7.4 and centrifuged for 10 min at 2,000 rpm. Reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) level were determined by protocols followed by earlier researchers.

**Reduced glutathione level**: For determining the GSH level, 10% tissue homogenate was mixed with 100 μl trichloroacetic acid (25%), centrifuged and the precipitates collected. Then, 200 μl of coloring reagent, 0.6 mM 5, 5’ dithio-bis-2-nitrobenzoic acid was added in 0.2 molar phosphate buffer solution having a pH of 8.0 up to 100 μl. The absorbance of nonprotein sulphahydryl (SH) group was calculated by ultraviolet-VIS Systronics spectrophotometer at 412 nm wavelength. Reduced glutathione level was determined in μmol/g of tissue (Moron et al., 1979).

**Catalase level**: For determining the catalase (CAT) level, 10%
tissue homogenate was mixed with 50 mM phosphate buffer solution having a pH 7. After 10 min centrifugation, 30 mM hydrogen superoxide solution was added in obtaining 100 μl solution. The absorbance was recorded by spectrophotometer at 240 nm. Catalase level was shown in μmol of H₂O₂ reduction/mg protein/min (Aebi, 1984).

**Superoxide dismutase level:** For determining the superoxide dismutase (SOD) leve, pyrogallol was added in 50 mM Tris-hydrochloride buffer solution having a pH 7.5. After auto oxidation, change in absorbance was calculated by spectrophotometer at 420 nm. Superoxide dismutase level was determined in μmol/g of protein (Marklund and Marklund, 1974).

**Lipid peroxidation level:** Lipid peroxidation (LPO) level was calculated by 0.6% active substances of thiobarbituric acid, 0.1% sodium dodecyl sulphate and 20% trichloroacetic acid. Then 200 μl 10% tissue homogenate were dissolved in these reagents and warmed this mixture for one hour. After cooling it, extraction was made with N butanol- pyridine in a ratio of 15:1 and the optical density values noted by spectrophotometer at 532 nm. All the results were expressed in nmol/mg of tissue (Ohkhawa et al., 1979).

**Histopathological study**

Specimens of mice ulcerated skin was excised washed with normal saline and fixed in 10% formalin for a day. Again it was fixed with paraffin wax, 5 micrometer portions of each specimen was cut and the histopathology observed (Parmar et al., 2011).

**Chromatogram by HPLC for identification of active constituent:** High performance liquid chromatography (HPLC) was performed to confirm the presence of quercetin in *C. arvensis* methanol extract (Ali et al., 2013). The sample was dissolved in 5 ml distilled water and 12 ml methanol, kept for 5 min, again to it was added 6 ml distilled water, allowed to stay for 5 min and again was added 10 ml 5 M HCl in this solution. It was placed in oven for 2 h and the solution filtered by syringe filter. Acetonitrile, methanol and acetic acid were used as the mobile phase with the flow rate of 1 ml/min. The column was ODS 250 mm × 4.6 mm and UV detector was used to obtain chromatogram at 280 nm at room temperature (Saleem et al., 2014).

**RESULTS**

All the obtained results were statistically analyzed by one way analysis of variance (ANOVA). Minitab 16.0 software was used for calculation and all values were represented as mean ± standard deviation (SD). Values were taken as p < 0.05 (significant). According to this screening, the methanol extract of *C. arvensis* had shown the presence of phytochemicals including: reducing sugars, alkaloids, tannins, saponins, terpenoids, flavonoids, anthraquinons and glycosides (Table 1).

**Morphological parameters**

Results obtained from present study had shown that single topical application of carcinogen DMBA followed by a thrice/week repeated application of 1% Croton oil till 16th week produced 100% skin ulceration in the carcinogen control group. 18.20 ± 1.643, 3.640 ± 0.3286 and 3.640 ± 0.3286 were calculated as the cumulative number of papilloma, tumor yield and tumor burden, respectively (Table 1 and Figure 1a and b). Ulcerated skin specimens were observed under microscope after staining with hematoxylin, a basic dye and eosin, an acidic die, as shown in Figure 2.

*Convulvulus arvensis* (methanol) extract in 300 mg/kg was applied locally to pre, peri, post and continuous groups. Significant decline in cumulative number of papillomas from pre to continuous group 15.800 ± 2.387 to 4.800 ± 6.111, tumor yield 3.160 ± 0.4775 to 0.9600 ± 1.322 and tumor burden 3.640 ± 0.329 to 1.200 ± 1.653 were calculated (Table 2). The tumor incidence decreased from 5 to 4 that is, 20% in continuous group compared with carcinogen group. When the results of the experimental group vs. carcinogen group was statistically analyzed, they showed a significant difference between groups (p < 0.05).

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Methanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing sugars</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>+</td>
</tr>
<tr>
<td>Anthra-quinones</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table 1.** Preliminary phytochemistry of whole plant methanol *Convulvulus arvensis* extract.
Figure 1. Morphologically representing papillomas on mice skin

Table 2. Inhibition of morphological parameters of DMBA/Croton oil induced skin tumors by *Convolvulus arvensis* (methanol) extract in 300 mg/kg topically applied dose.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Pre</th>
<th>Peri</th>
<th>Post</th>
<th>Continuous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor incidence</td>
<td>5.000 ± 0.000</td>
<td>5.000 ± 0.000</td>
<td>4.000 ± 2.236</td>
<td>4.000 ± 2.236</td>
<td>4.000 ± 2.236</td>
</tr>
<tr>
<td>Cumulative no. of papillomas</td>
<td>18.20 ± 1.643</td>
<td>15.800 ± 2.387</td>
<td>10.600 ± 6.229</td>
<td>8.600 ± 5.030</td>
<td>4.800 ± 6.111</td>
</tr>
<tr>
<td>Tumor yield</td>
<td>3.640 ± 0.3286</td>
<td>3.160 ± 0.4775</td>
<td>2.120 ± 1.2458</td>
<td>1.7200 ± 1.0060</td>
<td>0.9600 ± 1.3221</td>
</tr>
<tr>
<td>Tumor burden</td>
<td>3.640 ± 0.329</td>
<td>3.160 ± 0.477</td>
<td>2.650 ± 1.557</td>
<td>2.150 ± 1.257</td>
<td>1.200 ± 1.653</td>
</tr>
</tbody>
</table>

Each value was the mean of 3 readings and expressed as mean ± SD; Significance level between carcinogen control vs experimental groups (p<0.05).

Table 3. Induction of reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and inhibition of lipid peroxides (LPO) level by *C. arvensis* (methanol) extract in 300 mg/kg topically applied dose.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Pre</th>
<th>Peri</th>
<th>Post</th>
<th>Continuous</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>3.286 ± 0.207</td>
<td>4.3020 ± 0.449</td>
<td>5.5100 ± 0.1283</td>
<td>7.1260 ± 0.4953</td>
<td>7.1260 ± 0.4953</td>
</tr>
<tr>
<td>SOD</td>
<td>1.722 ± 0.1262</td>
<td>2.562 ± 0.5731</td>
<td>4.3300 ± 0.5665</td>
<td>5.3600 ± 0.2835</td>
<td>6.5160 ± 0.3710</td>
</tr>
<tr>
<td>CAT</td>
<td>13.624 ± 0.813</td>
<td>13.624 ± 0.813</td>
<td>14.240 ± 2.073</td>
<td>15.900 ± 0.900</td>
<td>18.792 ± 0.714</td>
</tr>
<tr>
<td>LPO</td>
<td>7.652 ± 0.1863</td>
<td>6.188 ± 0.2417</td>
<td>4.4760 ± 0.2370</td>
<td>4.5620 ± 0.2782</td>
<td>4.2340 ± 0.5928</td>
</tr>
</tbody>
</table>

Each value was the mean of 3 readings and expressed as mean ± SD; Significance level between carcinogen control vs experimental groups (p<0.05).

Biochemical parameters

Results obtained from present study had shown that topical application of DMBA and Croton oil produced 100% skin ulceration in carcinogen control group and decreased the GSH, SOD and CAT level to 3.286 ± 0.207 μmol/g, 1.722 ± 0.1262 μmol/g and 13.624 ± 0.813 μmol of H\textsubscript{2}O\textsubscript{2} reduction/mg protein/min levels, respectively and increased the LPO level as 7.652 ± 0.1863 nmol/mg (Table 3). *C. arvensis* (methanol) extract in 300 mg/kg caused a 20% decline in the tumor incidence in the continuous group. GSH, SOD and CAT increased up to 7.1260 ± 0.4953 μmol/g, 6.5160 ± 0.3710 μmol/g and 18.792 ± 0.714 μmol of H\textsubscript{2}O\textsubscript{2} reduction/mg protein/min level while LPO level decreased up to 4.2340 ± 0.5928 nmol/mg as compared with a carcinogen (Table 3). When the results of experimental vs. carcinogen group was statistically analyzed and compared they showed a significant difference between groups (p < 0.05). The results were supported by HPLC chromatogram as shown in Figure 3.

DISCUSSION

Cancer chemoprevention by conventional means or
Figure 2. Histopathology of C. arvensis methanol extract. (I) Carcinogen control: epidermis show ulceration and inflammatory slough; (II) Pre group: epidermis show mild acanthosis, hyperkeratosis and mild papillomatosis with normal cytological features; (III) Peri group: epidermis show mild papillomatous changes; (IV) Post group: epidermis show mild acanthosis with normal cytological features and (V) Continuous group: epidermis show very mild degree of acanthosis with normal cytological features.

Figure 3. HPLC Chromatogram for analysis of Convolvulus arvensis methanol extract.

Phytochemicals is capturing interests very rapidly. These chemical constituents include flavonoids, polyphenols, carotenoids, terpinoids and tannis which have been obtained from our daily dietary agents. They reverse the carcinogenesis and inhibit the development of persistent tumor (Sengupta et al., 2004). When tumor initiator, DMBA and tumor promoter, Croton oil (active constituent: 12-O-tetradecanoylphorbol-13-acetate) is applied on the mice skin, reactive oxygen species are formed. These ROS including O₂⁻, OH⁻, H₂O₂ have ability to move from
site of formation of the other healthy cells. DMBA with its active metabolites cause mutation of healthy cells via diol epoxide induction (Rubin, 2001). Increase ROS disturb the balance of oxidation/reduction reaction, oxidative stress parameters and take part in chemical carcinogenesis by changing the gene expression and destructing the cellular components. TPA along with ROS, increases the epidermal ornithine decarboxylase, COX-2 and nitric oxide synthase level (Shakilur et al., 2008).

Enzymatic oxidative stress parameters including SOD and CAT and non enzymatic GSH help to play important role in the enzymatic defense system and their lower level promote the tumor in healthy cells. Reduced glutathione helps to protect the body from xenobiotics, toxic metabolites and ROS (Lu, 1999). SOD and CAT capture the reactive oxygen species and minimize their carcinogenic and mutagenic potential, balance the hydrogen/oxygen peroxide level by causing alteration in O$_2$ and H$_2$O$_2$ radicals (Dasgupta et al., 2004). In carcinogen control group, level of GSH, SOD and CAT were significantly decreased and LPO increased along with the tumor incidence, tumor yield and tumor burden due to the presence of increased ROS. The whole plant of C. arvensis methanol extract decreased the tumor incidence, tumor yield, tumor burden, cumulative number of papilloma and lipid peroxidation level as compared to the carcinogen control group. This plant extract by two-folds increased the level GSH, SOD and CAT level in a continuous group in which plant extract was applied throughout the experimental period (16 weeks) at 300 mg/kg/b.wt. The phytochemical analysis had shown the presence of flavonoids, saponins, tannins and terpinoids and HPLC confirmed the querectin, that is flavonoid. Previous literatures have proved that querectin has ability to capture the ROS, superoxide anions, hydroxyl and lipid perox radicals, inhibit cyclooxygenase, lipoxygenase, monoxygenase, phospholipase A, protein kinase and NADH-oxidative pathways (Morton et al., 2000).

It has been reported and proved that the major constituent as tumor suppressor is flavonoids. Its cytotoxic activity is due to its unique structure. Activity of flavonoids is monitored by quantity, location and substitution of OH group or groups on A and B rings and double bond at C2-C3 level. Its activity is also connected with DNA topoisomerase II, which induce the destruction between DNA double stands and helps to reconnect these which performed various important functions in the cell mechanisms (Cao et al., 1996). Studies depicted that querectin, an important flavonoid having significant anti carcinogenic activity by inhibiting the over expression of tumor causing genes, functions of topoisomerase II, arrest the uncontrolled cell growth at G1, S, G2 and M in all phases of cell cycle and maintains its balance. Along with these it indirectly increases the level of tumor suppressor genes and their related genes and protein expression (Parmar et al., 2009). It is concluded that all those agents which have the ability to capture the free radicals in vivo may have cytotoxic potential against cancer.

Conclusion

C. arvensis methanol extract in 300 mg/kg has shown significant cytotoxic properties due to the presence of flavonoids and tannins against DMBA induced skin carcinogenesis. This investigation has introduced new phytochemicals for cancer prevention and will open new ways in this research era.

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Conflict of interest

There is no conflict of interest as regard this study.

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Arya P, Kumar M (2011). Chemoprevention by Triticum Aestivum of carcinogenesis. This investigation 


Review

Gastroretentive drug delivery systems: A review

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Oral administration has only limited use for important drugs from various pharmacological categories, that have poor oral bio-availability due to incomplete absorption and/or degradation in the gastrointestinal tract (GIT). Drugs that are easily absorbed from the gastrointestinal tract (GIT) and have a short half-life are eliminated quickly from the blood circulation, so they require frequent dosing. To avoid this drawback, the oral sustained-controlled release formulations have been developed in an attempt to release the drug slowly into the gastrointestinal tract (GIT) and maintain an effective drug concentration in the serum for longer period of time. However, such oral drug delivery devices have a physiological limitation of gastric retention time (GRT), variable and short gastric emptying time can result in incomplete drug release from the drug delivery system (DDS) in the absorption zone (stomach or upper part of small intestine), leading to diminished efficacy of the administered dose. The goal of any drug delivery system is to provide a therapeutic amount of drug to the proper site in the body to achieve promptly and then maintain the desired drug concentration. This idealized objective points to the two aspects most important to the drug delivery; namely spatial placement and temporal delivery of a drug. Spatial placement relates to targeting a drug to a specific organ or a tissue while temporal delivery refers to controlling the rate of drug delivery to that specific organ or a tissue.

Key words: Gastroretentive, drug delivery, controlled.

INTRODUCTION

Oral administration is the most convenient mode of drug delivery and is associated with superior patient compliance as compared to other modes of drug intake (Hofman A et al.,2004). Approximately 50% of the drug delivery systems available in the market are oral drug delivery systems which have more advantages due to patient acceptance and ease of administration(Das, 2000). The oral absorption of drugs is often limited due to short gastric retention time (GRT), that is, the time required for the content of the stomach to enter small intestine(Shargel and Andrew,1999). Drugs that are easily absorbed from the GIT and have a short half-life are eliminated quickly from the blood circulation, so they require frequent dosing. To avoid this drawback, the oral sustained-controlled release formulations have been developed in an attempt to release the drug slowly into the GIT and maintain an effective drug concentration in the serum for longer period of time (Ma et al., 2008). However, such oral drug delivery devices have a physiological limitation of gastric retention time (GRT), variable and short gastric emptying time can result in incomplete drug release from the drug delivery system.

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(DDS) in the absorption zone (stomach or upper part of small intestine), leading to diminished efficacy of the administered dose (Chaio and Robinson, 1995).

The goal of any drug delivery system is to provide a therapeutic amount of drug to the proper site in the body to achieve promptly and then maintain the desired drug concentration. This idealized objective points to the two aspects most important to the drug delivery, namely: spatial placement and temporal delivery of a drug. Spatial placement relates to targeting a drug to a specific organ or a tissue while temporal delivery refers to controlling the rate of drug delivery to that specific organ or a tissue (Lee and Robinson, 2000). Controlled drug release technology represents one of the frontier areas of science which involves multidisciplinary scientific approach, contributing to human health care. These drug delivery systems have a great potential of solving the problems associated with the conventional multiple dosing systems like strict adherence to timeliness of dosing, flip-flop plasma concentrations, associated side effects due to systemic accumulation of drug and patient non-compliance. Thus, there are numerous advantages such as improved efficacy, reduced toxicity, improved patient compliance and convenience etc. (Lalla, 1991).

An appropriately designed sustained or controlled release drug delivery system can be a major advance toward solving the problems associated with the existing drug delivery systems (Khan, 2001). Thus, a number of approaches are being developed. The common thread running through the approaches is the concept of self-administered, targeted, controlled release systems with increased bio-availability. However, incomplete release of the drug and a shorter residence time of dosage forms in the upper gastrointestinal tract, a prominent site for absorption of many drugs, will lead to lower bio-availability. Efforts to improve oral drug bio-availability have grown in parallel with the pharmaceutical industry. As the number and chemical diversity of drugs has increased, new strategies are required to develop orally active therapeutics. The past two decades have been characterized by an increased understanding of the causes of low bio-availability and a great deal of innovation in oral delivery technologies, marked by an unprecedented growth of the drug delivery industry (Orellana and Isabel, 2005). Thus, gastro retentive dosage forms which prolong the residence time of the drugs in the stomach and improve their bio-availability have been developed.

Oral delivery of drugs is by far the most preferable route of drug delivery due to ease of administration, patient compliance and flexibility in formulation. Development of oral controlled-release systems has been a challenge to formulation scientists because of their inability to restrain and localize the system in the targeted area of the gastrointestinal tract. Controlled/sustained release preparations using alternative routes have been formulated but the oral route still remains preferable.

When the drug is formulated with a gel forming polymer such as semisynthetic derivatives of cellulose, it swells in the gastric fluid with a bulk density less than one. It then remains buoyant and floats in the gastric fluid, and prolongs GRT (Patel et al., 2006).

Single-unit formulations are associated with problem being obstructed in the gastrointestinal tract, which may have a potential danger of producing irritation. On the other hand, a floating system made of multiple unit forms has relative merits compared to a single-unit preparation. On each subsequent gastric emptying, sunk particles will spread out over a large area of absorption sites, increasing the opportunity for drug release profile and absorption in a more or less predictable way. Moreover, since each dose consists of many sub-units, the risk of dose dumping is reduced (Gadad et al., 2009).

Gastraic emptying of dosage form is an extremely variable process and its ability to prolong and control the emptying time is valuable asset for dosage forms, which reside in the stomach for a long period of time than conventional dosage forms. Several difficulties are faced in designing controlled released systems for better absorption and enhance the bio-availability. Conventional oral dosage forms such as tablets, and capsules provide specific drug concentration in systemic circulation without offering any control over drug delivery and also cause great fluctuations in plasma drug levels. Although single-unit floating dosage forms have been extensively studied, these single-unit dosage forms have the disadvantage of a release all or nothing during emptying process while the multiple unit particulate system pass through the GIT to avoid the vagaries of gastric emptying and thus release the drug more uniformly. The uniform distribution of these multiple unit dosage forms along the GIT could result in more reproducible drug absorption and reduced risk of local irritation; this gave birth to oral controlled drug delivery and led to development of gastro retentive floating microspheres. This floating dosage form enhance bio-availability, having a dissolution and/or stability problem in the small intestine fluids, being locally effective in the stomach, being absorbed only in the stomach and/or upper part of the intestine (Sharma and Pawar, 2006).

STOMACH

An overview

The stomach is located in the upper left-hand portion of the abdomen just below the diaphragm (Tortora and Grabowski, 2000). It occupies a portion of the epigastric and left hydrochondriac region. The main function of the stomach is to store the food temporarily, grind it and then release it slowly into the duodenum. Due to its small surface area, very little absorption takes place from the stomach (Ross and Wilson, 2001).
Table 1. pH range of various organs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>pH range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>1-3</td>
</tr>
<tr>
<td>Small intestine</td>
<td>5-7.5</td>
</tr>
<tr>
<td>Large intestine</td>
<td>7.9-8</td>
</tr>
<tr>
<td>Rectum</td>
<td>7.5-8</td>
</tr>
</tbody>
</table>

Structure

The stomach has four main regions. The main function of fundus and body is storage, whereas that of cardia is mixing or grinding. The fundus adjusts the increased volume during eating by relaxation of the fundus muscle fibers. The fundus also exerts a steady pressure on the gastric contents pressing them towards the distal stomach. To pass through the pyloric valves into the small intestine, particles should be of size of 1 to 2 mm called chime. Anatomy of stomach is shown in Figure 1.

Functions of stomach

The stomach carries three major functions. It stores food, digest food and delivers food to the small intestine at a rate that the small intestine can handle.

1. Acts as a reservoir for holding food before it release into the small intestine.
2. Secrete gastrin into the blood.
3. Secretes gastric juice, which contains hydrochloric acid, pepsin, intrinsic factor and gastric lipase.
4. Mixes food and gastric juice to form chyme.

The pH range is shown in Table 1.

Regulation of gastric secretion and motility

Both neural and hormonal mechanisms control the secretion of gastric juice and the contraction of smooth muscles in the stomach wall. Events in gastric secretion occur in three overlapping phases; cephalic phase, gastric phase and intestinal phase.

Cephalic phase

The cephalic phase refers to the influence of the brain on secretion. Even before food enters the stomach, the sight, taste or thought of food initiate this phase, the secretion is brought about through stimulation of the nerve. This leads to presence of acid and pepsin in the stomach even before food enters the stomach (Saravanan et al., 2004).

Gastric phase

The gastric phase of secretion is brought about by the presence of food in the stomach. It is controlled by the hormone gastrin which is produced in the mucosa of the pyloric region of the stomach. Gastrin is released in response to stretching of the antrum caused by the presence of food in this region or in response to specific substances in the food; particularly proteins, alcohol and coffee are also potent stimulants of gastrin release. Once released, the gastrin is transported through the blood to stomach where it stimulates the secretion of hydrochloric acid and pepsinogen (Costa and Lobo, 2001).

Intestinal phase

The intestinal phase of acid secretion refers to the influence of the small intestine on gastric secretion. If the material present in the duodenum of the small intestine is too acidic, a hormone is released by the intestinal mucosa. This hormone is carried out by the blood to the body of the stomach where it inhibits further acid secretion. This serves as a protective device for the small intestine which is not as well protected against acid as the stomach. The total volume of gastric secretion in response to all the stimuli mentioned above is approximately 2 to 3 L per day (Kulkarni et al., 2004).

Gastric emptying

The process of gastric emptying occurs both during fasting and fed states. However, the pattern of motility differs markedly in the two states. In the fasted state, it is characterized by an inter-digestive cycle both through the stomach and small intestine, every 2 to 3 h. This activity is called the inter-digestive myoelectric cycle or migrating myoelectric complex (MMC). It is composed of four phases (Arora et al., 2005). The activities during gastric emptying is shown in Table 2.

1. Phase I (basal phase) lasts from 40 to 60 min with rare contractions.
2. Phase II (preburst phase) lasts for 40 to 60 min with intermittent action potential and contractions. As the phase progresses the intensity and frequency also increases gradually.
3. Phase III (burst phase) lasts for 4 to 6 min. It includes intense and regular contractions for short period. It is due to this wave that all the undigested material is swept out of the stomach down to the small intestine. It is also known as the housekeeper wave.
4. Phase IV lasts for 0 to 5 min and occurs between phases III and I of 2 consecutive cycles.

Factors affecting gastric retention

Gastric residence time of an oral dosage is affected by several factors.
Table 2. Activities during phases of gastric emptying.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I (basal phase)</td>
<td>Period of rare contraction lasting from 40 to 60 min</td>
</tr>
<tr>
<td>Phase II (pre-burst phase)</td>
<td>Period intermittent contraction and of similar duration of 60 min</td>
</tr>
<tr>
<td>Phase III (burst phase)</td>
<td>Period of regular contraction at the maximal frequency lasting from 4 to 6 min</td>
</tr>
<tr>
<td>Phase IV</td>
<td>Period of transition between phase III and Phase I and last for 0 to 5 min</td>
</tr>
</tbody>
</table>

Figure 1. Anatomy of stomach.

1. Volume of meal: the larger the bulk of meal, the longer will be the gastric emptying time.
2. Composition of meal: fats promote the secretion of bile, which has an inhibitory effect on gastric emptying time.
3. Physical state of food and dosage form: viscous material empty slowly than less viscous materials.
4. Exercise: Retards gastric emptying time.
5. Emotion: Stress and anxiety promotes gastric motility whereas as depression retards it.
6. Circadian rhythms: Cardiac rhythms are increased in day time and less during night also affect the gastric retention time.
7. Size of the dosage form: Greater the energy content of meal (carbohydrate and high fat content), longer the duration of emptying.
8. Density of oral dosage form: The density of gastric fluid is reported to be 1.2g/cm³. The density of the dosage form should be less than this for buoyancy so that it is retained in the stomach for longer period of time.
9. Diseased state: state of the stomach also affects the environment for the dosage form as in case of ulcers, flatulence and spasms (Brahmankarand Jaiswal, 2006).
10. Drug therapy: It also plays an important role in gastric emptying e.g. prokinetic drugs like cisapride and mosapride increase gastric emptying time whereas imipramine and atropine retards it.
11. Age: Increase in age decreases gastric motility there by increasing the gastric emptying time.
12. Posture: Gastric emptying is favored while standing and by lying on right side since normal curvature of the stomach provides a downhill path whereas lying on the left side or in supine position retards it.

GASTRO RETENTIVE DRUG DELIVERY SYSTEMS (GRDDS)

It is evident from the recent scientific and patent literature that an increased interest in novel dosage forms that are retained in stomach for a prolonged and predictable period of the time exists today in academic and industrial research groups (Garg and Gupta, 2008).

Criteria for selection of drug candidate for GRDDS

The gastro retentive drug delivery systems are suitable for following types of drug therapy:

1. Drugs those are locally active in the stomach e.g.
miroprostol, antacids etc.
2. Drugs that have narrow absorption window in gastrointestinal tract (GIT) for example, LDOPA, paraaminobenzoic acid, furosemide, riboflavin etc.
3. Drugs that are unstable in the intestinal or colonic environment e.g. captopril, ranitidine HCl, metronidazole.
4. Drugs that disturb normal colonic microbes e.g. antibiotics against Helicobacter pylori.
5. Drugs that exhibit low solubility at high pH values e.g. diazepam, chlordiazepoxide, verapamil HCl (Nayak et al., 2010).

Advantages of GRDDS

1. Enhanced bio-availability.
2. Reduced frequency of dosing.
3. Targeted therapy for local ailments in the upper GIT.
4. Patient compliance.
5. Improved therapeutic efficacy (Singh and Kim, 2000).

Gastro-retentive drug delivery system (GRDDS) greatly improves pharmacotherapy of the stomach through local drug release leading to high drug concentrations at gastric mucosa (eradicating helicobacter pylori from the sub mucosal tissue of the stomach), making it possible to treat stomach and duodenal ulcers, gastritis, and esophagitis, reduce the risk of gastric carcinoma, controlled release antacid formulations. GRDDS can be used as carriers for drugs which are absorbed from absorption windows in stomach. For example various antibiotics (amoxicillin, ampicillin, tetracyclines, amoxicillin; sulphonamides, quinolones, penicillins, cephalosporins, aminoglycosides and tetracyclines, etc.) are taken up only from very specific sites of the GI mucosa.

Disadvantages of GRDDS

There are certain situations where gastric retention is not desirable. Aspirin and non-steroidal anti-inflammatory drugs are known to cause gastric lesions and slow release of such drugs in the stomach is unwanted. Thus drugs that may irritate the stomach lining or are unstable in its acidic environment should not be formulated in gastro retentive systems. Furthermore, other drugs such as isosorbidinedinitrate that are absorbed equally well throughout the GIT will not be suitable for incorporation into a gastric retention system (Deshpande et al., 1997). Also GRDD’s have some limitations such as:

1. Requirement of high levels of fluids in stomach for the delivery system to float and work efficiently.
2. Requires the presence of food to delay gastric emptying.
3. Drugs, which undergo significant first pass metabolism, may not be desirable candidates for floating drug delivery system since the slow gastric emptying.
4. May lead to alter systemic bioavailability.
5. Drugs having solubility or stability problems in the highly acidic gastric environment or which are irritants to gastric mucosa cannot be formulated as GRDDS.

On the other hand, violent gas generation, disintegration of dosage forms, burst release, dose dumping and alkaline micro-environment are the limitations of floating alginate beads. In case of bio-adhesive systems, the acidic environment, thick mucus as well as high turnover rate of mucous prevents bond formation at the mucous polymer interface. For swell-able systems, the dosage form must maintain a size larger than the aperture of the resting pylorus for required time period.

Applications of GRDDS

Floating drug delivery offers several applications for drugs having poor bioavailability because of the narrow absorption window in the upper part of the gastrointestinal tract. It retains the dosage form at the site of absorption and thus enhances the bioavailability (Shirwalkar et al., 2006) Marketed products of GRDDS are shown in Table 4.

APPRAOCHES TO GASTRIC RETENTION

A number of approaches have been used to increase GRT of a dosage form in stomach by employing a variety of concepts. These Includes.

Sustained drug delivery

Hydro-dynamically balanced systems (HBS) can remain in the stomach for long periods and hence can release the drug over a prolonged period of time. The problem of short gastric residence time encountered with an oral controlled release formulation hence can be overcome with these systems. These systems have a bulk density less than 1 as a result of which they can float on the gastric contents. These systems are relatively large in size and passing from the pyloric opening is prohibited (Mayavanshi and Gajjar, 2008).

Site specific drug delivery

These systems are particularly advantageous for drugs that are specifically absorbed from stomach or the proximal part of the small intestine, for example riboflavin and furosemide.

Absorption enhancement

Drugs that have poor bio-availability because of site
specific absorption from the upper part of the gastrointestinal tract are potential candidates to be formulated as floating drug delivery systems, thereby maximizing their absorption (Deepa and Karthikeyan, 2009). This is illustrated in Figure 2.

**Floating systems**

Floating drug delivery systems (FDDS) have a bulk density lower than gastric fluids and thus remain buoyant in stomach for a prolonged period of time, without affecting the gastric emptying rate. While the system floats on gastric contents, the drug is released slowly at a desired rate from the system. After the release of drug, the residual system is emptied from the stomach. This results in an increase in gastric retention time and a better control of fluctuations in plasma drug concentrations. Floating systems can be classified into two distinct categories, no effervescent and effervescent systems (Rajinikanth et al., 2008).

**Bio/muco-adhesive systems**

Bio/muco-adhesive systems are those which bind to the gastric epithelial cell surface or mucin and serve as a potential means of extending gastric residence time of drug delivery system in stomach, by increasing the intimacy and duration of contact of drug with the biological membrane. Binding of polymers to mucin/epithelial surface can be divided into three broad categories, name; Hydration-mediated adhesion bonding-mediated adhesion and receptor-mediated adhesion.

**Swelling and expanding systems**

These are dosage forms, which after swallowing, swell to an extent that prevents their exit from the pylorus. As a result, the dosage form is retained in stomach for a long period of time. These systems may be named as “plug typesystem”, since they exhibit tendency to remain logged at the pyloric sphincter (Fursule et al., 2008) Various types of floating drug delivery systems are shown in Table 3.

**High density systems**

These systems with a density of about 3 g/cm³ are retained in the rugae of stomach and are capable of withstanding its peristaltic movements. A density of 2.6 to 2.8 g/cm³ acts as a threshold value after which such

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**Table 3. Various types of floating drug delivery systems**

<table>
<thead>
<tr>
<th>Dosage forms</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tablets</td>
<td>Acetaminophen, acetylsalicylic acid, amoxicillin, atenolol, chlorpheniramine, diltiazem, Isosorbidenmonitrate,Prednisolone,Theophylline, Sotalol, Prednisolone, Quinidine gluconate,Piretanide</td>
</tr>
<tr>
<td>Capsules</td>
<td>Diazepam,furosemide,misoprostol,benserazide,L-Dopa</td>
</tr>
<tr>
<td>Films</td>
<td>Cinnarizine</td>
</tr>
<tr>
<td>Granules</td>
<td>Diclofenac sodium,indomethacin,prednisolone</td>
</tr>
<tr>
<td>Microspheres</td>
<td>Aspirin,ibuprofen,griseofulvin,tranilast</td>
</tr>
<tr>
<td>Powders</td>
<td>Basic drugs</td>
</tr>
</tbody>
</table>
systems can be retained in the lower parts of the stomach. High density formulations include coated pellets. Coating is done by heavy inert material such as barium sulphate, zinc oxide, titanium dioxide, iron powder etc.

Incorporation of passage delaying food agents

Food excipients like fatty acids, for example salts of myristic acid change and modify the pattern of stomach to a fed state, thereby decreasing gastric emptying rate and permitting considerable prolongation of release. The delay in gastric emptying after meals rich in fats is largely caused by saturated fatty acids with chain length of C10 to C14 (Rokhadeet et al., 2007).

Ion exchange resins

Ion exchange resins are loaded with bicarbonate and a negatively charged drug is bound to the resin. The resultant beads are then encapsulated in a semi permeable membrane to overcome the rapid loss of carbon dioxide. Upon arrival in the acidic environment of the stomach, an exchange of chloride and bicarbonate ions take place. As a result of this reaction carbon dioxide is released and trapped in the membrane thereby carrying beads towards the top of gastric content and producing a floating layer of resin beads in contrast to the uncoated beads, which will sink quickly.

Osmotic regulated systems

It is comprised of an osmotic pressure controlled drug delivery device and an inflatable floating support in a bio-erodible capsule. In the stomach the capsule quickly disintegrates to release the intra-gastric osmotically controlled drug delivery device. The inflatable support inside forms a deformable hollow polymeric bag that contains a liquid that gasifies at body temperature to inflate the bag. Osmotic system is shown in Figure 4. The osmotic controlled drug delivery device consists of two components: drug reservoir compartment and osmotically active compartment (Srivastava et al., 2005).

FLOATING DRUG DELIVERY SYSTEMS (FDDS)

Based on the mechanism of buoyancy, two distinctly different technologies have been utilized in development of FDDS which are; effervescent system and non-effervescent system. Intragastric floating drug delivery devices are shown in Figure 7.

Effervescent systems

These buoyant delivery systems are prepared with swellable polymers such as methocel or polysaccharides for example, chitosan and effervescent components, e.g. sodium bicarbonate and citric or tartaric acid or matrices containing chambers of liquid that gasify at body temperature. The matrices are fabricated so that upon contact with gastric fluid, carbon dioxide is liberated by the acidity of gastric contents and is entrapped in the gelled hydrocolloid. This produces an upward motion of the dosage form and maintains its buoyancy (Arora et al., 2005). The effervescent systems are classified into:

Multiple-unit oral floating drug delivery system

Recently a multiple-unit type of floating pill, which generates carbon dioxide gas, has been developed (Figure 3). The system consisted of sustained release pills as seeds surrounded by double layers. The inner layer is an effervescent layer containing both sodium bicarbonate and tartaric acid. The outer layer was a
swellable membrane layer containing mainly polyvinyl acetate and purified shellac. Moreover, the effervescent layer was divided into two sub layers to avoid direct contact between sodium bicarbonate and tartaric acid. Sodium bicarbonate was contained in the inner sub layer and tartaric acid was in the outer layer. When the formed swollen pills, like balloons, with a density much lower than 1.004 g/cm$^3$. The reaction was due to carbon dioxide generated by neutralization in the inner effervescent layer with the diffusion of water through the outer swellable membrane layer (Yeole, 2005).

The system was found to float completing within 10 min and approximately 80% remained floating over a period of 5 h irrespective of pH and viscosity of the test medium. A floating system utilizing ion-exchange resins have been developed. The system consisted of resin beads, which were loaded with bicarbonate and a negatively charged drug that was bound to the resin. The resultant beads were then encapsulated in a semi permeable membrane to overcome rapid loss of carbon dioxide. Upon arrival in the acidic environment of stomach, an exchange of chloride and bicarbonate ion took place, as was expected. As result of this reaction, carbon dioxide was released and trapped in the membrane, thereby carrying beads toward the top of gastric contents and producing a floating layer of resin beads. In contrast, the uncoated beads sank quickly. Radioactivity measurement by scintigraphy showed that gastric residence was substantially prolonged, compared with a control, when the system was given after a light, mainly liquid meal. Furthermore, the system was capable of slow release of drug. A property which widens the scope of such floating system for SR preparation of drugs possessing negative charge since they can be easily bound to the resin in combination with bicarbonate ions. Two patents on FDDS issued to the Alza Corporation disclosed drug delivery devices for the controlled and continuous administration of medicinal agents (Garg and Sharma, 2003).

**Inflatable gastrointestinal drug delivery system**

The residence time of the drug delivery device in the stomach can also be sustained by incorporation of an inflatable chamber, which contains a liquid, e.g. ether that gasifies at body temperature to cause the chamber to float in the stomach.

**Intragasricosmotically controlled drug delivery system**

It is comprised of an osmotic pressure controlled drug delivery and an inflatable floating support in a bioerodible capsule. When the drug delivery device reaches the site of drug administration e.g. the stomach, the capsule quickly disintegrates to release the
Intragastricosmotically controlled drug delivery device. The inflatable floating support is made from a deformable hollow polymeric bag that contains a liquid that gasifies at body temperature to inflate the bag. This system is shown in Figure 7.

**Osmotic controlled drug delivery system**

Although single unit floating dosage forms have been extensively studied, these single unit dosage forms have the disadvantage of a release all or nothing emptying process while the multiple unit particulate system pass through the GIT to avoid the vagaries of gastric emptying and thus release the drug more uniformly. The uniform distribution of these multiple unit dosage forms along the GIT could result in more reproducible drug absorption and reduced risk of local irritation; this gave birth to oral controlled drug delivery and led to development of gastro-retentive floating microsphere (Vyasand Khar, 2002).

**Non-effervescent systems**

Commonly used excipients, here are gel-forming or highly swell-able cellulose type hydrocolloids, polysaccharides and matrix forming polymers such as polycarbonate, polyacrylate, polymethacrylate and polystyrene. One of the approaches to the formulation of such floating dosage forms involves intimate mixing of drug with the gel-forming hydrocolloid, which swells in contact with gastric fluid after oral administration and maintains a relative integrity of shape and a bulk density of less than unity within the outer gelatinous barrier. The air entrapped by the swollen polymer confers buoyancy to these dosage forms. The gel structure acts as a reservoir for sustained drug release as the drug is slowly released by controlled diffusion through the gelatinous barrier. Non-effervescent systems include the following:

1. Hydrodynamically balanced intragastric delivery system.
2. Bilayer tablets.
3. Intragastric floating gastro intestinal drug delivery system.
4. Hollow/floating microspheres.

**Hydrodynamically balanced intragastric delivery system (HBS)**

The hydrodynamically balanced gastrointestinal drug delivery systems, in either capsule or tablet form, is designed to prolong GI residence time in an area of the GI tract to maximize drug reaching its absorption site in solution state and hence, ready for absorption. It is prepared by incorporating a high level (20 to 75% w/w) of one or more gel-forming hydrocolloids, for example hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose and sodium carboxymethylcellulose into the formulation and then compressing these granules into a tablet (or encapsulating into capsules). On contact with gastric fluid the hydrocolloid in this intragastric floating device start to become hydrated and forms a colloidal gel barrier around its surface with thickness growing with time. This gel barrier controls the rate of solvent penetration into the device and the rate of drug release from the device (Indian Pharmacopoeia Commission, 2007). It maintains a bulk density of less than 1 and thus remains buoyant in the gastric fluid inside the stomach for up to 6 h. System is illustrated in Figure 5.

**Principle of hydrodynamically balance system**

**Bilayer tablet**

A bilayer tablet can be prepared to contain one immediate-release layer and one sustained-release layer. After the initial dose is delivered by the immediate release layer, the sustained release layer absorbs the gastric fluid and forms a colloidal gel barrier on its surface. This produces a bulk density less than that of the gastric fluid and remains buoyant in the stomach for extended period of time (Altaf et al., 2008). Bilayer tablet is shown in Figure 6.

**Intragastric floating gastro intestinal drug delivery system**

A gastrointestinal drug delivery system (GIDS) can be made to float in the stomach by incorporating a floatation chamber, which may be a vacuum or filled with a harmless gas (Rahman et al., 2006). A drug reservoir is encapsulated inside a microporous compartment with apertures along its top and bottom walls. The peripheral walls of the drug reservoir compartment are completely sealed to prevent any direct contact of the stomach mucosal surface with the undissolved drug. In the stomach the floatation chamber causes the GIDS to float in the gastric fluids. Fluids enter through the apertures, dissolve the drug, and carry and drug solute out of the drug delivery system for controlled transport to the intestine for absorption (Bolourtchian et al., 2008).

**Hollow/floating microspheres**

Floating microspheres are gastro retentive drug delivery systems based on non-effervescent approach. Hollow microspheres are in strict sense, spherical empty particles without core. These microspheres are
characteristically free flowing powders consisting of proteins or synthetic polymers, ideally having a size less than 200 micrometer. Solid bio-degradable microspheres incorporating a drug dispersed or dissolved throughout particle matrix have the potential for controlled release of drugs (Xuet et al., 2006). Gastro retentive floating microspheres are low density systems that have sufficient buoyancy to float over gastric contents and remain in stomach for prolonged period. As the system floats over gastric contents, the drug is released slowly at desired rate resulting in increased gastric retention with reduced fluctuations in plasma drug concentration (Gryllaki et al., 2007). Preparation of floating microspheres by solvent evaporation method is shown in Figure 8.

**List of polymers used in hollow microspheres**

Cellulose acetate, chitosan, eudragit, acrycoat, methocil,
polyacrylates, polyvinyl acetate, carbopol, agar, polyethylene oxide, polycarbonates, acrylic resins and polyethylene oxide (Gupta et al., 2007).

**Advantages of hollow microspheres**

1. Improves patient compliance by decreasing dosing frequency.
2. Bioavailability enhances despite first pass effect because fluctuations in plasma drug concentration is avoided, a desirable plasma drug concentration is maintained by continuous drug release.
3. Gastric retention time is increased because of buoyancy.
4. Enhanced absorption of drugs which solubilize only in
stomach
5. Drug releases in controlled manner for prolonged period.
6. Site-specific drug delivery to stomach can be achieved (Basavaiah and Prameela, 2004).
7. Superior to single unit floating dosage forms as such microspheres releases drug uniformly and there is no risk of dose dumping.
8. Avoidance of gastric irritation, because of sustained release effect.
9. Better therapeutic effect of short half-life drugs can be achieved (Reymond et al., 1986).

LIMITATIONS
Floating drug delivery system requires sufficiently high level of fluids in stomach for the drug delivery to float and to work efficiently. It is not suitable for those drugs that have solubility or stability problems in gastric fluids. Drugs such as Nifedipine, which is well absorbed along the entire GI tract and which undergoes significant first-pass metabolism may not be desirable candidates for FDDS, since the slow gastric emptying may lead to reduce systemic bioavailability of FDDS for drugs that are irritant to gastric mucosa (Nilkumhang and Basit, 2009).

Methods of preparation of hollow microspheres
Hollow microspheres are prepared by solvent diffusion and evaporation methods to create the hollow inner core. Polymer is dissolved in an organic solvent and the drug is either dissolved or dispersed in the polymer solution. The solution containing the drug is then emulsified into an aqueous phase containing polyvinyl alcohol to form oil in water emulsion. After the formation of a stable emulsion, the organic solvent is evaporated either by increasing the temperature under pressure or by continuous stirring. The solvent removal leads to polymer precipitation at the o/w interface of droplets, forming cavity and thus making them hollow to impart the floating properties (Basavaraj et al., 2008).

Mechanism of floating microspheres
When microspheres come in contact with gastric fluid the gel formers, polysaccharides, and polymers hydrate to form a colloidal gel barrier that controls the rate of fluid penetration into the device and consequent drug release. As the exterior surface of the dosage form dissolves, the gel layer is maintained by the hydration of the adjacent hydrocolloid layer. The air trapped by the swollen polymer lowers the density and confers buoyancy to the microspheres. However a minimal gastric content needed to allow proper achievement of buoyancy. Hollow microspheres of acrylic resins, eudragit, polyethylene oxide, and cellulose acetate; polystyrene floatable shells; polycarbonate floating balloons and gelucire floating granules are the recent developments (Mastiholimath et al., 2007).

CONCLUSION
The goal of any drug delivery system is to provide a therapeutic amount of drug to the proper site in the body and also to achieve and maintain the desired plasma concentration of the drug for a particular period of time. However, incomplete release of the drug, shorter residence times of dosage forms in the upper GIT leads to lower oral bio-availability. Such limitations of the conventional dosage forms have paved way to an era of controlled and novel drug delivery system.

Conflict of interest
Authors have none to declare.

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Full Length Research Paper

Synthesis and antifungal activities of some benzimidazolyl-chalcones, analogues of chlormidazole

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We report here the synthesis of new benzimidazolyl-chalcones analogues of chlormidazole in order to contribute to the development of new antifungal drugs. Benzimidazolyl-chalcones were obtained by reaction of various aromatic aldehydes (7) with N-(4-chlorobenzyl)-2-acetylbenzimidazole (5) and its precursor 2-acetylbenzimidazole (4). After confirming their structure using spectroscopic methods (1H and 13C NMR, MS in EI mode), the compounds were evaluated for their antifungal activities against a clinical strain of Candida albicans in order to determine the minimum inhibiting quantity (MIQ). This screening showed that compounds 6e, 6f and 6h had anti-Candida efficiencies (MIQ = 5, 1.25 and 0.625 µg) higher than those of chlormidazole (MIQ = 10 µg). Additionally it has shown that improving these activities in benzimidazolyl-chalcones series requires double chemical modulation: Removal of 4-chlorobenzyl on the pyrrole nitrogen of the benzimidazole ring and the introduction or not of modulators such as fluorine (compounds 6f and 6h) on the benzene ring at position 3 of the propenone.

Key words: Benzimidazole, chalcone, chlormidazole, antifungal, Candida albicans.

INTRODUCTION

The candidosic infections are diseases due to the development of Candida yeasts in human. Candida albicans species, the most known and the most dangerous (Develoux and Britain, 2005) can cause serious illnesses that can result in high mortality and increased hospitalization costs especially in immunosuppressed patients (HIV, diabetes, cancers etc.) (Morgan et al, 2005; Lass-Flörl, 2009; Pfaller and Diekema, 2007). Therapeutic support management of these infections constitutes a public health issue with emergence of drug resistant strains to most of the current antifungals [Djohan et al., 2012; Bryskier, 1999; Sanglard and Odds, 2002]. This drug resistance is more alarming with azoles antifungals which are the most commonly...
The objective of this work is to synthesize and select new antifungal drug candidates in benzimidazolyl-chalcones series. Specifically, it is for us to determine in this chemical series, the minimal quantities capable to inhibit the proliferation of *C. albicans* and to establish favorable structural elements for antifungal activities.

**MATERIALS AND METHODS**

**Chemistry**

Preparation of benzimidazolyl-chalcones derivatives was carried out in two major steps: the first step was to prepare *N*-(4-chlorobenzyl)-2-acetylbenzimidazole (5) and the second consisted of preparing benzimidazolyl-chalcones (6a-d).

**Synthesis of *N*-(4-chlorobenzyl)-2-acetylbenzimidazole (5)**

Synthesis of compound 5 was carried out using the reaction sequence illustrated in Scheme 1. It started with a condensation according to Phillips method (Phillips, 1928) between orthophenylenediamine (1) and lactic acid (2). The reaction proceeded at reflux in dilute hydrochloric acid. This step led after neutralization with ammonia to 2-hydroxyethyl benzimidazole (3). Compound (3) was then oxidized by potassium dichromate in acetic acid afforded to 2-acetylbenezimidazole (4) after neutralization with ammonia. *N*-chlorobenzylolation of compound (4) in presence of sodium hydride gave *N*-(4-chlorobenzyl)-2-acetylbenzimidazole 5.

**Synthesis of benzimidazolyl-chalcones 6a to j**

*N*- (4-chlorobenzyl)-2-acetylbenzimidazole 5 previously prepared
and its precursor 4, were engaged in a Claisen-Schmidt condensation with various substituted benzaldehydes 7. This reaction led after neutralization by dilute acetic acid to the expected benzimidazolyl-chalcones 6a to j (Scheme 2). All benzimidazolyl-chalcones derivatives and their precursors were isolated in powder form and characterized by nuclear magnetic resonance (1H and 13C NMR) and mass spectrometry in electron impact mode (EI). Moreover, their melting points were determined on a Kofler bench. As for chlormidazole used in this work as reference antifungal drug and molecular model, it was obtained by total synthesis.

Antifungal activities

To evaluate antifungal activities of all compounds, we used the clinical strain of *C. albicans* 27506 provided by the Centre de Diagnostic et de Recherche sur le SIDA et les Maladies Opportunistes (CeDReS) of CHU Treichville in Abidjan, Cote d'Ivoire. The antymycotic screening method used was the bioautography or “agar overlay” (Homans and Fuchs, 1970; Rahalison, 1994; Rahalison et al., 1994; Rahalison et al., 1991). It is a method to determine *in vitro* the minimum inhibiting quantity (MIQ) fungal growth by thin-layer chromatography (TLC). The products were first solubilized in methanol in order to prepare the stock solutions containing 1 mg/ml. From each of these mother solutions, was prepared a range of 10 dilutions. Thereafter, 10 ml of each solution were deposited on TLC plate. Chromatograms were developed in a saturated mobile phase CHCl₃·CH₃OH·H₂O in a ratio (65: 35: 5) and then dried. An inoculums containing approximately 105 cells/ml of *C. albicans* was obtained by inoculation of three colonies of a pure strain for 24 to 48 h in a tryptone soya broth. These inoculums were subsequently spread on each chromatogram. Plates were first incubated at 30°C after solidification of the agar for 24 h and then impregnated with an aqueous solution methylthiazolyl tetrazolium chloride (MTT). Finally, after incubation for 2 to 4 hours, the growth inhibition zones subsequently appeared as white spots on a purple background. Only products that showed an inhibition zone were selected to determine their Minimum Inhibiting Quantities.

RESULTS

Chemical results

We synthesized and isolated 10 chalcones derivatives (Table I) carring a benzimidazole in their phenylpropenone moiety. These compounds were divided in two series: the N-4-chlorobenzyl derivatives (compounds 6a to 6e) and the non-N-substituted derivatives (compounds 6f to 6j). Moreover, benzene ring in position 3 of the propenone in both series carried various modulators like (Cl, F) and nitro (NO₂) (Figure 2). The spectroscopic NMR proton characterization (Table 1) of benzimidazolyl-chalcones showed three characteristic peaks: from 5.91 to 5.95 ppm for NCH2Ph, from 7.86 to 8.15 ppm for H2 and from 8.01 to 8.40 ppm for H3. As for the 13C spectra, we noted four main peaks: from 47.76 to 48.25 ppm NCH2Ph, from 121.69 to 123.01 ppm for C2, from 136.55 to 144.99 ppm for C3, from 180.00 to 182.72 ppm for C = O. The molecular peaks in Mass Spectrometry of these new chalcones (Table I) varied between 267 and 452 depending on their substituents.

Antifungal activities results

The results of anti- *Candida albicans* screening in Table 2 showed that the strain CeDReS had sensitivity for the various products tested at different concentrations. Thus chlormidazole presented anticandidosic activities of 10 µg at the limit of our experimentation. It is the same for the benzimidazolyl-chalcones 6a, analogue of chlormidazole and six derivatives of this compound. As against three other derivatives that is, compounds 6e, 6f and 6h, they were able to induce much better anticandidosic activities, respectively at 5, 1.25 and 0.625 µg.

DISCUSSION

Pharmacochemical method of apposition of multiple pharmacophores entities allowed to establish that the replacement of the 2-methyl of chlormidazole by phenylpropenone led to a new chalcone (compound 6a) having anticandidosic activities (MIQ =10 µg). These activities were superimposed to those of chlormidazole. Tests to improve activities of compound 6a by pharmacomodulation (Figure 3) showed that the introduction of modulators like halogen (Cl, F) or nitro on the benzene ring in position 3 (compounds 6b to 6d) did not improve the anti- *Candida* activities which were superposable to those of compound 6a and chlormidazole (MIQ = 10 µg). But the concomitant presence of chlorine and nitro on the benzene ring (compound 6e) was suitable to the improvement of anti- *Candida* activities with a MIQ of 5 µg. This anti- *Candida* efficiency was 2 times higher than those of benzimidazolyl-chalcone 6a and chlormidazole.

The removal of N-4-chlorobenzyl in compound 6a gave compound 6f, surprisingly, this improved anticandidosic activities with a MIQ of 1.25 µg. This antifungal efficacy was 8 times higher than those of its N-substituted analogue (compound 6a) and chlormidazole. Taking chalcone derivative 6f as reference, tests to improve anticandidosic activities by the introduction of chlorine and/or nitro group (compounds 6g, 6i and 6j) into its benzene ring was not satisfactory. Indeed the anti- *candida* activities of these compounds remained at 10 µg like those of N-4-chlorobenzyl derivatives 6a to d. However, the presence of fluoride doubled the removal of N-4-chlorobenzyl (compound 6h) led to the exaltation of anticandidosic efficiency with MIQ of 0.625 µg. In fact, the derivative 6h was, respectively 2 and 16 times more effective than those of 6f and 6a. If the non substitution of pyrrole nitrogen of the benzimidazole showed positive
Table 1. Physicochemical characteristics of 6a to j compounds.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Physicochemical characteristics</th>
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<tbody>
<tr>
<td>6a</td>
<td>RMN $^1$H: 8.29 (1H, d, $J = 15.8$ Hz, CH=CH); 7.92 (1H, d, $J = 15.8$ Hz, CH=CH); 5.94 (2H, s, CH$_2$). RMN $^{13}$C: 182.72 (C=O); 144.99 (CH=CH); 122.84 (CH=CH); 48.20 (CH$_2$). SM: 373 ([M$^+$], 100). Purification: column chromatography (eluent: hexane / dichloromethane: 30/70). Yield = 73%. MP = 150°C</td>
</tr>
<tr>
<td>6b</td>
<td>RMN $^1$H: 8.29 (1H, d, $J = 15.8$ Hz, CH=CH); 7.92 (1H, d, $J = 15.8$ Hz, CH=CH); 5.95 (2H, s, CH$_2$). RMN $^{13}$C: 182.72 (C=O); 141.86 (CH=CH); 123.01 (CH=CH); 47.76 (CH$_2$). SM: 407 ([M$^+$], 80). Purification: column chromatography (eluent: hexane / dichloromethane: 50/50). Yield = 40%. MP &gt; 260°C</td>
</tr>
<tr>
<td>6c</td>
<td>RMN $^1$H: 8.25 (1H, d, $J = 15.8$ Hz, CH=CH); 7.86 (1H, d, $J = 15.8$ Hz, CH=CH); 5.93 (2H, s, CH$_2$). RMN $^{13}$C: 182.95 (C=O); 127.45 (CH=CH); 122.02 (CH=CH); 48.25 (CH$_2$). SM: 391 ([M$^+$], 100). Purification: column chromatography (eluent: hexane / dichloromethane: 30/70). Yield = 79%. MP = 168°C</td>
</tr>
<tr>
<td>6d</td>
<td>RMN $^1$H: 8.40 (1H, d, $J = 15.8$ Hz, CH=CH); 8.09-7.95 (4H, m, CH=CH et H$_\alpha$); 5.94 (2H, s, CH$_2$). RMN $^{13}$C: 182.38 (C=O); 137.46 (CH=CH); 122.04 (CH=CH); 48.25 (CH$_2$). SM: 417 ([M$^+$], 60). Purification: washing in hot hexane; Yield = 62%. MP= 140°C</td>
</tr>
<tr>
<td>6e</td>
<td>RMN $^1$H: 8.39 (1H, d, $J = 15.8$ Hz, CH=CH); 8.10-7.95 (4H, m, CH=CH et H$_\alpha$); 5.94 (2H, s, CH$_2$). RMN $^{13}$C: 183.01 (C=O); 136.68 (CH=CH); 122.03 (CH=CH); 48.25 (CH$_2$). SM: 452 ([M$^+$], 35). Purification: washing in hot hexane; Yield = 50%. MP = 150°C</td>
</tr>
<tr>
<td>6f</td>
<td>RMN $^1$H: 8.28 (1H, d, $J = 16$ Hz, CH=CH); 8.15 (1H, d, $J = 16$ Hz, CH=CH). RMN $^{13}$C: 181.13 (C=O); 143.97 (CH=CH); 121.69 (CH=CH). SM: 249 ([M$^+$], 100). Purification: recrystallization from toluene. Yield = 54%. MP = 216°C</td>
</tr>
<tr>
<td>6g</td>
<td>RMN $^1$H: 8.01 (1H, d, $J = 15$ Hz, CH=CH); 7.96 (1H, d, $J = 15$ Hz, CH=CH). RMN $^{13}$C: 180.00 (C=O); 142.25 (CH=CH); 122.01 (CH=CH). SM: 283,72 ([M$^+$], 100). Purification: recrystallization from ethanol/toluene: 1/4. Yield = 47%. MP&gt;260°C</td>
</tr>
<tr>
<td>6h</td>
<td>RMN $^1$H: 8.01 (1H, d, $J = 15$ Hz, CH=CH); 7.91 (1H, d, $J = 15$ Hz, CH=CH). RMN $^{13}$C: 180.80 (C=O); 142.55 (CH=CH); 122.01 (CH=CH). SM: 267 ([M$^+$], 100). Purification: recrystallization from toluene. Yield = 81%. MP = 222°C</td>
</tr>
<tr>
<td>6i</td>
<td>RMN $^1$H: 8.39 (1H, d, $J = 15.9$ Hz, CH=CH); 8.10-7.85 (2H, m, CH=CH et H$_\alpha$). RMN $^{13}$C: 182.38 (C=O); 137.40 (CH=CH); 122.04 (CH=CH). SM: 294 ([M$^+$], 100). Purification: recrystallization from ethanol/toluene: 1/1. Yield = 49% MP= 200°C</td>
</tr>
<tr>
<td>6j</td>
<td>RMN $^1$H: 8.39 (1H, d, $J = 15.9$ Hz, CH=CH); 8.10-7.85 (2H, m, CH=CH et H$_\alpha$). RMN $^{13}$C: 183.01 (C=O); 136.58 (CH=CH); 122.09 (CH=CH). SM: 328 ([M$^+$], 100). Purification: recrystallization from ethanol/toluene: 1/1. Yield = 45%. MP = 208°C</td>
</tr>
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Scheme 1. Synthesis of N-(4-chlorobenzyl)-2-acetylbenzimidazole 5
Scheme 2. Synthesis of benzimidazolyl-chalcones 6a to j.

Figure 2. Structure of benzimidazolyl chalcones synthesized.

Figure 3. Structural variations of compound 6a.
impact on anthelmintic activities (AFECT, 1998), it also seems to be essential for induction of antifungal activities.

Finally, in the series of benzimidazolyl-chalcones, induction and enhancement of antifungal activities passed through a double modulation namely the deletion of the 4-chlorobenzyl on the pyrrole nitrogen of the benzimidazole nucleus and the modulation or not of the homocycle benzene at position 3 of the propenone by a fluorine.

Conclusion

Research of new antifungal in helping to fight against mycotic diseases allowed us to conceptualize and synthesize a new series of benzimidazolyl-chalcones. Evaluation of antifungal activities against C. albicans of these benzimidazolyl-chalcones showed that the induction and the enhancement of these activities were due to a double chemical modulation. This is the removal of 4-chlorobenzyl on pyrrole nitrogen of the benzimidazole ring and the introduction or not of modulator such as fluorine on the homocycle benzene at position 3 of the propenone. Accordingly, compounds 6f and 6h having, respectively MIQ of 1.25 and 0.625 µg were able to completely inhibit the growth of C. albicans.

These compounds could be the leaders of a new class of total synthesis antifungal.

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Conflict of interest

Authors have none to declare.

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Antitoxic effect of baicalin and glycyrrhetic acid on PC12 cells induced by *Aconitum brachypodum* Diel

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The roots of *Aconitum brachypodum* Diel are used clinically in traditional medicine of China. However, it is also found very toxic and to date, few detoxication strategies are available to completely eliminate its toxicity. The present study was conducted to observe the cellular neurotoxicity of *A. brachypodum* Diels and discuss the detoxication effect of baicalin and glycyrrhetic acid, two compounds from Chinese herbal Baical skullcap root and Radix Glycyrrhizae, respectively. The cellular neurotoxicity of *A. brachypodum* Diels on PC12 cell was estimated and assessed. The results showed that low doses of chloroform-soluble fraction (CFA) displayed little toxicity on PC12 cells. However, long term of high doses (300 to 400 μg/ml) exposure to CFA could lead to significant cell damage, mainly including cell apoptosis and necrosis, increasing intracellular reactive oxygen species (ROS), mitochondria dysfunction and redox imbalance. Pre-incubation of baicalin and glycyrrhetic acid could both decrease the cytotoxicity of PC12 induced by CFA. The present study strongly demonstrated the pro-oxidant effects of CFA and suggested that increased intracellular ROS and calcium have mediated a significant time and dose-dependent cytotoxicity in PC12 cells exposed to CFA via a mitochondrial dependent pathway, which could be effectively reduced by baicalin and glycyrrhetic acid.

**Key words:** *Aconitum brachypodum* Diel, baicalin, glycyrrhetic acid, PC12, detoxication.

**INTRODUCTION**

*Aconitum brachypodum* Diels belong to the genus of Aconitum (Family: Ranunculaceae). Its root is used clinically as an anti-rheumatic, anti-inflammatory and anti-nociceptive drug in traditional medicine of China (Ren et al., 2012; Huang et al., 2013). However, it is also very toxic due to aconitine alkaloids, especially aconitine, the main alkaloid of this plant. The toxic effects of aconitine and its structurally related analogs are known to affect mainly the heart and the central nervous system (AMERI, 1998). In case of overdoses, acute poisoning or intoxication resulting from misuse, instant treatment including the careful monitoring of vital signs (blood pressure, arrhythmia, etc.) is crucial because of the rapid deterioration of the patient’s condition in the first 24 h. However, the treatment can only be supportive since there is no antidote (Chan, 2009). So far, few detoxication strategies are available to completely neutralize the toxicity of *A. brachypodum* Diels. Its neurotoxicity and pharmacological mechanism need further investigation.
Some Chinese herbal drugs can help to relieve aconite-poisoning (Singhuber et al., 2009). For example, Radix Glycyrrhizae (Gancao) is a commonly used detoxifying herb in traditional Chinese medicine (TCM). The combined use of Radix Glycyrrhizae and Radix Aconiti is a safe pair in TCM formulations (Gu et al., 2008). By combining with Aconitum and Glycyrrhiza (mostly in decoction), the toxicity of Aconitum can be largely reduced (Ma et al., 2006; Noguchi et al., 1978). The decoction with Radix Glycyrrhizae decreases LD50 of Fuzi to an undetectable value (Lei et al., 2007).

The concept of "hot/cold" has been used in TCM to describe the "energies" of medicine, foods and drinks. The concept is derived from the Yin/Yang in Daoism: hot being Yang (阳) and cold being Yin (阴). Any Chinese herbs can be roughly classified into hot, cold and neutral categories. Yin/Yang theory also indicates that "cold" medicine can be used to neutralize or decrease the toxicity induced by "hot" medicine, and vice versa.

Since A. brachypodum Diels belong to "hot" medicine, "cold" medicine should be used to reduce its toxicity. Baicalin, the major bioactive constituent of the isolated root of Scutellaria baicalensis, is also a common "cold" medicine in TCM. It is widely used in China and Southeast Asian countries (Ma et al., 2009). Evidence has indicated that baicalin has anti-apoptotic (Cheng et al., 2012), anti-oxidant (Cao et al., 2011), anti-tumor, anti-ischemic, anti-inflammatory (Guo et al., 2013) and immune system modulatory activities (Li et al., 2010). Baicalin has been reported to play a beneficial role in various experimental models of invasive toxicants by alleviating inflammatory injury (Kim and Lee, 2012) via the involvement of TLR2- or TLR4-mediated innate immune reactions (Hou et al., 2012; Li et al., 2012), and thus was selected to observe the detoxication effect on A. brachypodum Diels.

The present study was conducted to observe the cellular neurotoxicity of A. brachypodum Diels and discuss its pharmacological mechanism. Moreover, baicalin and glycyrrhetic acid, two compounds from Baical skullcap root and Radix Glycyrrhizae, respectively, were used to observe their detoxication effects.

MATERIALS AND METHODS

Extract preparation

The dried roots of A. brachypodum Diels were bought from Bozhou city, Anhui province in China. The plant was authenticated by Dr. Xinqiao Liu, Associate Professor in Pharmacognosy at School of Pharmacy, South-central University for Nationalities. The dried roots of A. brachypodum Diels were grinded into powder and submerged in 95% ethanol and left to macerate for three times. The combined solution was filtered and evaporated to complete dryness using a standard Buchi rotary-evaporator. The 95% ethanolic extract was dissolved in 2% vitriol and degreased with petroleum ether. Ammonia was used to regulate pH value to 9.0. The majority of the ethanolic extract was suspended with water and successively extracted with chloroform.

In a pilot investigation, the chloroform-soluble fraction (CFA) (Figure 1) was the most toxic fraction among the extractions and was selected for this study. The CFA was subjected to column
chromatography and reversed-phase high performance liquid chromatography (HPLC). The apparatus used for HPLC was Ultimate3000 (Thermo Fisher, USA). The conditions were as follows: column, a YMC-Pack ODS-A column (4.6 × 250 mm, YMC, Japan); mobile phase, methanol - 0.1% triethylamine (0 to 15 min 13.7 [v/v], 16 to 45 min 39:11 [v/v]). Four aconitine alkaloids were identified by comparing their retention time with those of the reference standards (Figure 1).

Reagents

3-(4, 5 - Dimethylthiazol -2 - yl ) – 2, 5 –diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemicals Co (St Louis, Missouri, USA). Fluo-3 AM was purchased from Biotium (USA). 7'- dichlorodihydrofluorescin diacetate (DCFH-DA) was purchased from Sigma Chemicals Co (St Louis, Missouri, USA). 5, 5', 6, 6'-tetrachloro-1', 3 ', 3'- tetraethyl benzimidazol carbocyanine iodide (JC-1, Molecular Probes) was purchased from Beyotime Institute of Biotechnology, China. Dopamine (DA) assay was from Shanghai-Jianglai Institute (China). Roswell Park Memorial Institute-1640 (RPMI-1640) was purchased from Hyclone (Logan, UT). Fetal bovine serum (FBS) was purchased from HYCLONE Life Technologies. Baicalin and glycyrrhetic acid were purchased from National Institutes for Food and Drug Control (China). All chemicals were of the highest purity commercially available.

Cell culture and drug treatment

The rat pheochromocytoma cell line PC12 were obtained from China Center for Type Culture Collection. The cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated horse serum, 5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a water-saturated atmosphere of 5% CO2 at 37°C. The culture medium was changed every other day and cells were subcultured about three times a week. All experiments were carried out 12 h after cells were seeded at an appropriate density according to each experimental scale. The CFA was freshly prepared with dimethyl sulfoxide (DMSO) and diluted by culture media at the desired concentrations just before use. The PC12 cells were preincubated with indicated concentration of CFA for various period of time. In a pilot investigation, cells were treated with CFA at concentrations ranging from 0 to 400 µg/ml for various intervals (6, 12, 18, 24 h) and then examined for cell viability. Concentration of 200 µg/ml CFA as control was used in an extensive study of detoxification of baicalin and glycyrrhetic acid. Baicalin (1 to 9 µM) or glycyrrhetic acid (5 to 40 µM) were added 2 h before CFA was added.

Cell viability assay

The cell viability was assessed by using the MTT assay as previously described (Li et al., 2013). Briefly, PC12 cells (1 × 10^5 cells/ml) were treated with CFA and baicalin or glycyrrhetic acid for 24 h at 37°C. After 3 h incubation with MTT (0.5 mg/ml), cells were lysed in DMSO and the amount of MTT formazan was qualified by determining the absorbance at 570 nm using a microplate reader (TECAN A-5082, meglan, AUSTRIA). Cell viability was expressed as a percent of the control value. Meanwhile, the concentration of the CFA used in assays of ROS and mitochondrial membrane potential were based on the results of the MTT test.

Measurement of DA release

The supernatant of PC12 cells was collected for the detection of DA release. The production of DA was measured by enzyme linked immunosorbent assay (ELISA) method using commercial kits (Jianglai Co. Ltd, Shanghai, China) according to the manufacturer's instructions.

Measurement of mitochondrial membrane potential (∆Ψm)

To assess ∆Ψm, a cell-permeable cationic and lipophilic dye, JC-1 was used as previously described (Cossarizza et al., 1993). This probe aggregates within mitochondria and fluoresces red (590 nm) at higher ∆Ψm. However, at lower ∆Ψm, JC-1 cannot accumulate within the mitochondria and instead remains in the cytosol as monomers, which fluoresce green (490 nm). Therefore, the ratio of red to green fluorescence gives a measure of the transmembrane electrochemical gradient. PC12 (10^5 cells) were treated with CFA and baicalin or glycyrrhetic acid for 24 h at 37°C. The cells were then incubated with JC-1 (5 µg/ml) in the dark for 15 min at room temperature. Then, cells were washed in phosphate-buffered saline (PBS), suspended in 400 µl of PBS and analyzed by flow cytometry.

Measurement of intracellular reactive oxygen species (ROS)

The generation of ROS for the cells was evaluated by a fluorometry assay using intracellular oxidation of DCFH-DA. The cells in logarithmic growth phase were incubated in a 6-well plates for 24 h for stabilization, then the medium was replaced with medium containing different concentrations (0-400 µg/ml) of CFA for 24 h. Different concentrations of baicalin (2.25, 4.5 µM) and glycyrrhetic acid (10, 20 µM) were pre-incubated with 200 µg/ml of CFA to observe their antitoxic effect. After exposure, the cells were washed with PBS, then they were re-suspended at a concentration of 1 × 10^5 cells/ml and were stained by the staining solution for 20 min, the cells were detected and analyzed by flow cytometry.

Measurement of intracellular calcium

Intracellular calcium level was determined with Fluo-3/AM. The green fluorescent levels reflecting intracellular calcium transient function were determined by flow cytometry. Briefly, the cells were washed with PBS after samples exposure described earlier, then they were re-suspended at a concentration of 1 × 10^5 cells/ml and were stained with 5 µM Fluo-3/AM (Biotium, USA) in RPMI-1640 and incubating at 37°C for 30 min. The fluorescence was measured and analyzed by flow cytometry.

Statistical analysis

Statistical analysis was performed using statistical package for social sciences (SPSS) 11.5 for windows. All results were presented as mean ± standard error of mean (SEM). Group differences were analyzed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD’s) post hoc tests. A probability of P < 0.05 was considered significant.

RESULTS

The dose- and time- response effect of CFA on PC12 cells

As shown in Figure 2, lower dosage (< 100 µg/ml) of CFA could enhance the proliferation of PC12 cells during a
Figure 2. Effect of CFA on cell viability of PC12. Cell viability was measured by MTT assay. Control value was taken as 100%. Values are expressed as mean ± SEM, n = 8. Significant statistical difference was indicated by *P < 0.05 **P < 0.01 vs. control group.

short period of 6 h. However, the cell viability was decreased when the concentration and time period increased. After 12 h of incubation, the viability of the cells significantly decreased under the concentration of 300 to 400 μg/ml. As time increased, significant cytotoxicity of CFA was observed at the concentrations of 200 to 400 μg/ml. ANOVA analysis and Dunnett’s test revealed that PC12 cells were inhibited both in a dose-dependent and time-dependent manner by CFA.

Inhibition of baicalin and glycyrrhetic acid on cell viability of CFA-injured PC12 cells

As shown in Figure 3, baicalin and glycyrrhetic acid either for 2 h prior to, or during the CFA insult, prevent the decreased cell viability in a dose-dependent manner, which indicated the neuroprotective effect of baicalin and glycyrrhetic acid on CFA-induced injury. Pre-incubation of baicalin and glycyrrhetic acid prior to CFA insult (Figure 3A) had stronger protective effect than co-incubation during the insult (Figure 3B). The cell viabilities were increased to (82.4 ± 1.6%) and (85.6 ± 2.9%) after co-incubation of baicalin (9 μM) and glycyrrhetic acid (40 μM), respectively. Otherwise, the cell viability were increased to (84.2 ± 3.9%) and (91.8 ± 3.8%), respectively after pre-incubation of them with the same concentration. Based on the results, pre-incubation of baicalin and glycyrrhetic acid were selected for detection of DA release, ΔΨm, intracellular ROS and calcium of insulted PC12 cells.

The effect of baicalin and glycyrrhetic acid on DA release

As shown in Figure 4, incubation of CFA (200 μg/ml) for 24 h could significantly evoke DA production from PC12 cells. Pre-incubation of baicalin (1 to 9 μM) and glycyrrhetic acid (5 to 40 μM) could both inhibit CFA-induced DA release of PC12 cells.

Inhibition of baicalin and glycyrrhetic acid on ΔΨm induced by CFA

As shown in Figure 5, ΔΨm decreased after 24 h of CFA treatment. In contrast, pre-treatment of baicalin and glycyrrhetic acid could both partially reverse the decrease of ΔΨm induced by CFA. These results indicated the implication of mitochondrial dysfunction in the pathogenesis of cell apoptosis and the sensitivity of ΔΨm towards mitochondrial ROS generated by CFA.

Inhibition of baicalin and glycyrrhetic acid on generation of intracellular ROS induced by CFA

As shown in Figure 6, there was significant increase of intracellular ROS after incubation of CFA (200 μg/ml) for
Figure 3. Inhibition of baicalin and glycyrrhetic acid on cell viability of CFA-injured PC12 cells. Cells were (A) co-incubation or (B) pre-incubation with baicalin and glycyrrhetic acid for 2 h, then CFA was added for 24 h. Significant statistical difference was indicated by **P<0.01 vs. control group, *P<0.05, ##P<0.01 vs. CFA group.

Figure 4. The effect of baicalin and glycyrrhetic acid on DA release induced by CFA. Cells were pre-incubation with baicalin or glycyrrhetic acid for 2 h, then CFA was added for 24 h. Significant statistical difference was indicated by *P<0.05 vs. Control group, **P<0.05, ***P<0.01 vs. CFA group.
Figure 5. Inhibition of baicalin and glycyrrhetic acid on $\Delta \Psi_m$ induced by CFA. Cells were pre-incubation with baicalin or glycyrrhetic acid for 2 h, then CFA was added for 24 h. (A) Control; (B) CFA 200 μg/ml; (C) baicalin 4.5 μM; (D) glycyrrhetic acid 20 μM. The figure shows an average of three experiments done independently.

Figure 6. Inhibition of baicalin and glycyrrhetic acid on generation of intracellular ROS induced by CFA. Cells were pre-incubation with baicalin or glycyrrhetic acid for 2 h, then CFA was added for 24 h. (A) control; (B) CFA 200 μg/ml; (C) CFA 200 μg/ml + baicalin 2.25 μM; (D) CFA 200 μg/ml + baicalin 4.5 μM; (E) CFA 200 μg/ml + glycyrrhetic acid 10 μM; (F) CFA 200 μg/ml + glycyrrhetic acid 20 μM. The figure shows an average of three experiments done independently.
24 h. Pre-incubation of baicalin and glycyrrhetic acid at different concentrations could both decrease the intracellular ROS induced by CFA to some extent.

The effect of baicalin and glycyrrhetic acid on intracellular calcium

As shown in Figure 7, there was significant increase of intracellular calcium after incubation of CFA (200 μg/ml) for 24 h. Pre-incubation of baicalin and glycyrrhetic acid at different concentration could both decreased the intracellular calcium induced by CFA to some extent.

DISCUSSION

The present results showed that lower dose of CFA displayed little toxicity on PC12 cells. However, long term exposure to 300 to 400 μg/ml of CFA could lead to cell apoptosis and necrosis, increasing DA production, increasing intracellular ROS, mitochondria dysfunction and redox imbalance (Figure 8).

DA, the neurotransmitter in DAergic neurons, can be spontaneously oxidized into electron-deficient DA quinone, which readily forms a covalent bond with nucleophiles, such as the thiol group on the amino acid cysteine (Graham et al., 1978). DA can be oxidized to generate semiquinones, quinones, oxygen radicals and other reactive oxygen species. These toxic molecules ultimately contribute to the inhibition of mitochondrial respiration and lipid peroxidation, which may play an important role in neuronal cell death (Chen et al., 2003; Zhao et al., 2010). Irreversible modification of cysteine residues on proteins can alter the function of the protein, potentially jeopardizing the health of the cell. Covalent modification of cysteiny1 residues forming 5-cysteinyl-dopamine in both proteins and glutathione side effects (GSH) is thought to be the mechanism underlying the toxicity of DA to the neurons (Hastings et al., 1996).

It is possible that free radical metabolites are responsible for the cytotoxicity observed in PC12 cells. Mitochondria are important producers of ROS, which can be highly damaging and inhibitory to cardiomyocyte function. Mitochondria also serve as calcium buffers, protecting neurons from excitotoxic cell death (Duchen, 2000). At the same time, mitochondria are the main targets of ROS-induced oxidative damage (Starkov, 2008; Saretzki, 2009). The mitochondrial-to mitochondrion ROS-induced ROS release constitutes a positive feedback mechanism for enhanced ROS production leading to potentially significant mitochondrial

**Figure 7.** The effect of baicalin and glycyrrhetic acid on intracellular calcium of PC12 induced by CFA. Cells were pre-incubation with baicalin or glycyrrhetic acid for 2 h, then CFA was added for 24 h. (A) control; (B) CFA 200 μg/ml; (C) CFA 200 μg/ml + baicalin 2.25 μM; (D) CFA 200 μg/ml + baicalin 4.5 μM; (E) CFA 200 μg/ml + glycyrrhetic acid 10 μM; (F) CFA 200 μg/ml + glycyrrhetic acid 20 μM. The figure shows an average of three experiments done independently.
The present study demonstrated that CFA-induced alterations in mitochondrial redox functions were critical in promoting ROS-mediated cell death. The decrease of ΔΨₘ induced by CFA showed a ROS-mediated mitochondrial dysfunction in the cells. Thus, the CFA-induced alterations in mitochondrial function in PC12 cells may be a major cause of apoptosis and cell death. The results showed that CFA-induced cell death was an effect of increased oxidative stress signaling and alterations in the mitochondrial redox. Baicalin and glycyrrhetic acid might improve mitochondrial function through eliminating the CFA-induced overproduction of ROS.

Calcium plays a pivotal role in many inter- and intraneuronal processes, including (dopaminergic) neurotransmission (Westerink, 2006), gene transcription (Carrasco and Hidalgo, 2006), neurodegeneration (Mattson, 2012), and neurodevelopment (Pravettoni et al., 2000). Arrhythmogenic toxicity of aconitine is related to intracellular Ca²⁺ signals (Zhou et al., 2013). Neuronal cells rely heavily on strict regulation of their intracellular calcium concentration ([Ca²⁺]). Increased mitochondrial oxidative stress was dependent on mitochondrial Ca²⁺ overload in PC12 cells, because blocking mitochondrial Ca²⁺ uptake prevented elevated superoxide production. In this study, the results also showed that in PC12 cells, CFA increased [Ca²⁺], and induced triggered activities such as ROS.

The present study demonstrated the pro-oxidant effects of CFA and suggested that increased intracellular ROS and calcium have mediated time and dose-dependent cytotoxicity in PC12 cells exposed to CFA via a mitochondrial dependent pathway. Baicalin and glycyrrhetic acid could effectively reduced CFA-induced neurotoxicity.

Conflict of interest

The authors declare that there is no conflict of interest.

ACKNOWLEDGMENTS

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REFERENCES

Full Length Research Paper

Effect of piperine on pentylenetetrazole induced seizures, cognition and oxidative stress in mice

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Cognitive impairment in epileptics may be a consequence of the epileptogenic process as well as antiepileptic medication. Thus, there is need for drugs, which can suppress epileptogenesis as well as prevent cognitive impairment. In the present study, the effect of piperine was evaluated on the course of pentylenetetrazole (PTZ) induced seizures, learning deficit and oxidative stress markers in mice. Male albino mice were injected with PTZ (65 mg/kg sc) on the 5th day of the treatment for the development of seizures. Spontaneous alternation behaviour (SAB) was carried out on the 1st and the 5th day of the treatment after PTZ administration, while the oxidative stress parameters (malondialdehyde and glutathione) were carried out in the whole brain upon the completion of the behavioural assessment. The administration of piperine, 2 mg/kg significantly decreased the PTZ induced seizures and showed improvement in the learning deficit induced by PTZ as evidenced by the increased latency time and frequency of jerks and improvement in spontaneous alternation behavior (SAB). The findings suggest the potential of piperine as adjuvant to antiepileptic drugs with an added advantage of preventing cognitive impairment.

Key words: Cognitive impairment, piperine, pentylenetetrazole.

INTRODUCTION

Epilepsy and antiepileptic drug therapy: Present status

Epilepsy is one of the oldest conditions known to mankind and still the most common neurological condition affecting individual of all ages. At any given time, it is estimated that 50 million individuals worldwide have a diagnosis of epilepsy. The prevalence is much higher in developing countries than in developed countries owing to low economic status and limited access to health care (Beghi and Hesdorffer, 2014; Banarjee et al., 2009). Our understanding of the pathophysiology of the epilepsies has advanced dramatically in the last 30 years, especially in terms of their cellular physiology and genetics. Drug treatment of epilepsy has made remarkable strides, with the introduction of many new antiepileptic drugs since 1978. Improvement in terms of clinical outcome however, has fallen short of expectations; with up to one third of patients continuing to experience seizures or unacceptable
side medication related side effects in spite of efforts to identify optimal treatment regimens with one or more drugs (Meldrum and Rogawski, 2007). There is an urgent need to identify the problems associated with drug therapy in epilepsy. Antiepileptic drug treatment may last a lifetime in many patients so the objective of treatment should be such as to attain the best compromise between maximum seizure control and minimum side effects. The current vastly improved understanding of the molecular targets, coupled with advances in the pathophysiology of epilepsy which include a succession of breakthroughs in genetics will lead to improved therapies for epilepsy.

Epilepsy, AEDs and cognition

Cognitive impairments are commonly seen in patients taking antiepileptic drugs (AEDs). For many patients, there may be more debilitating than the actual seizures themselves and thus, contribute to a worse quality of life. Common cognitive deficits in people with epilepsy are intellectual decline, reduced information processing speed, reduced reaction time, attention deficits and memory impairments (Menlove and Reilly, 2015; Mc Cagh et al., 2009; Fischer et al., 2000). The origin of such cognitive impairments has been attributed to several factors: a) the underlying etiology of epilepsy, b) the central side effects of taking antiepileptic drugs (AEDs), c) the effects of the seizures themselves and d) mood (Eddy et al., 2011; Mula and Trimble, 2009; Motamedi and Meador, 2004; Drane and Meador, 2002). Thus, while the underlying brain pathology, type, frequency and severity of seizures and psychological factors play an important role, ironically the therapy used also add to the problem (Arif et al., 2009). Most often, these factors are related and contribute in varying degrees to the cognitive profile of the individual patients. Of these factors, side effects associated with AED therapy may be one of the few potentially preventable tolerability issues, so it is worthwhile to further explore ways to prevent or minimize them. Therefore, induced cognitive impairments need pharmacological intervention.

Need of the hour: Drug therapy with maximum seizure control and minimum side effects

Modern antiepileptic therapy is neither universally effective nor invariably safe. Advancement in understanding pathophysiology of epilepsies in term of cellular physiology and genetics would allow for more judicious therapeutic approaches to this complex neurological disorder (White and Loscher, 2014; Jacob et al., 2009). Current practice suggests that combining drug with different mechanisms is likely to optimize the therapeutic response. Monotherapy may not be effective in all cases. Therefore, the need of the hour is to search for combinations of AEDs with other drugs so as to achieve supra-additive efficacy and infra-additive toxicity. Combinations of AEDs with nootropic agents appear to be promising directions for research in this area for maximal seizure control with minimal cognitive deficits. The adjuncts chosen for reducing cognitive impairments should be free of additional risk of side effects.

On the background of these observations, the present study was conducted to investigate the effect of piperine on pentylenetetrazole (PTZ) induced seizures, cognition and oxidative stress in mice. Piperine, a nitrogenous pungent substance, is an alkaloid presents in the fruits of black pepper (Piper nigrum), long pepper (Piper longum) and other piper species (family: Piperaceae). It is used as an important ingredient for various medicinal purposes in traditional systems of medicine. piperine reported to exhibit cognition enhancer and anticonvulsant properties (Bukhari et al., 2013; Chen et al., 2013; Saraogi et al., 2013; Chonpathompikulert et al., 2010; Wallanthorn et al., 2008), so an attempt was made to study its effect per se and in combination of SVP on PTZ induced seizures.

SVP was used as a reference drug for comparison and combination studies. This study was designed to look for a combination therapy for epilepsy that may help to achieve maximal efficacy with minimal side effects.

MATERIALS AND METHODS

Animals

Swiss strain adult male albino mice weighing between 18 to 25 g were used. The animals were housed in polypropylene cages in groups of 8 mice per cage and were kept under controlled environmental conditions (temperature: 22 to 28°C, natural light-dark cycle). The mice were maintained on a standard pellet diet and water ad libitum. Only active and apparently healthy animals with no visible lesions or gross abnormalities were selected for the experiments. All studies were conducted during the day time.

Drugs and dosing schedule

Pentylenetetrazole powder (Sigma, USA), sodium valproate powder (Sigma, USA) and piperine powder (Sigma, USA) were used in the study. All the drugs were dissolved in distilled water. The dose of sodium valproate (300 mg/kg) was selected on the basis of pilot experiments in our lab. This dose exhibited less than 50% protection against the chemoshock caused by PTZ (65 mg/kg, s.c.). Two doses of piperine (2 and 4 mg/kg) were used. All observations were made 90 min after sodium valproate (SVP) and 60 minutes piperine treatment. All drugs were given in a volume of 10 ml/kg. Control animals received the appropriate vehicle. The treatment schedule is given in Table 1. There were 6 groups, each having 6 mice. The animals were treated as per the given schedule.

PTZ-induced seizures

Pilot experiments were carried out to ascertain the dose of PTZ that produced convulsions in 100% of animals without mortality. This was found to be 65 mg/kg, s.c. The animals were observed
Table 1. Treatment schedule.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dosage, route of administration and duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NS</td>
<td>0.9% NaCl p.o., single dose for 5 days</td>
</tr>
<tr>
<td>B</td>
<td>PTZ</td>
<td>0.9% NaCl p.o., single dose for 4 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.9% NaCl p.o. + 65 mg/kg PTZ s.c. on 5th day</td>
</tr>
<tr>
<td>C</td>
<td>SVP</td>
<td>300 mg/kg S.V.P p.o., single dose for 4 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300 mg/kg S.V.P p.o. + 65 mg/kg PTZ s.c. on 5th day</td>
</tr>
<tr>
<td>D</td>
<td>P₁</td>
<td>2 mg/kg p.o. single dose for 4 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mg/kg p.o. + 65 mg/kg PTZ s.c. on 5th day</td>
</tr>
<tr>
<td>E</td>
<td>P₁</td>
<td>4 mg/kg p.o. single dose for 4 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 mg/kg p.o. + 65 mg/kg PTZ s.c. on 5th day</td>
</tr>
<tr>
<td>F</td>
<td>SVP + P₁</td>
<td>2 mg/kg Compound P₁ + 300 mg/kg S.V.P p.o. single dose for 4 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mg/kg Compound P₁ + 300 mg/kg S.V.P p.o. + 65 mg/kg PTZ s.c. on 5th day</td>
</tr>
</tbody>
</table>

NS: Normal saline; PTZ: Pentylenetetrazole; SVP: Sodium valproate; P₁: Piperine.

immediately after PTZ injection for a period of 30 min. The assessment was done following the method of Osonee et al. (1994). The latency to jerks, myoclonus and clonic generalized seizures with the loss of righting reflex was observed. In the absence of seizures within 30 min, the latency was taken as 1800 s.

Assessment of cognitive function

Spontaneous alternation behavior (SAB) on a cross maze

The method described by McIntyre et al. (1998) was followed. A wooden cross maze was used. Mice were placed individually on the central platform of the maze and were allowed to traverse the maze freely. The number and sequence of entries was noted during an observation period of 6 min. An alternation was defined as entry into four different arms on overlapping quintuple sets. Five consecutive arm choices within the total set of arm choices made up a quintuple set. A quintuple set consisting of arm choices B, A, C, B, D comprised an alternation while the set with B, A, D, B, A did not. Percent alternation was calculated as follows:

\[
\text{Alternation} (%) = \frac{\text{Actual number of alternations}}{\text{Possible number of alternations}} \times 100
\]

Where, possible alternations = number of arm entries minus 4. Memory was assessed on the cross maze before (1st day) and after (5th day) of the drug treatment.

Assessment of oxidative stress

At the end of the drug treatment schedule, the animals were killed under deep ether anaesthesia. Whole brain was removed and 10% tissue homogenates were prepared by separately homogenizing sufficient amounts of brain tissues in 0.15 M solution of potassium chloride (KCl). Homogenate was separated and used to determine protein content, malondialdehyde and glutathione. Protein content was estimated by the method described by Lowry et al. (1951). Malondialdehyde, an indicator of lipid peroxidation was estimated as described by Ohkawa et al. (1979) and glutathione was assessed by the method as described by Ellman (1959).

Statistical analysis

The data were expressed as mean ± standard error of mean (SEM). The results were analysed by a one-way analysis of variance (ANOVA) followed by a Dunnett’s t test or Mann-Whitney test, wherever appropriate.

RESULTS

PTZ induced seizures

SVP (300 mg/kg p.o.) pre-treatment for 5 days significantly increased the latency for the onset of jerks and myoclonus and clonic generalised seizures. It also significantly decreased the frequency of jerks. Piperine (2 and 4 mg/kg p.o.) significantly increased the latency for the onset of jerks and myoclonus and clonic generalised seizures. There was also a significant decrease in the frequency of jerks with these two doses of P₁. Concurrent administration of piperine (2 mg/kg p.o.) with SVP (300 mg/kg p.o.) significantly prolonged the latency to jerks and myoclonus and clonic generalised seizures. The frequency of jerks was also significantly decreased with this combination. All the comparisons were made with respect to the PTZ group (Table 2).

Cognitive function

Spontaneous alternation behavior (SAB) on a cross maze

The concomitant administration of P₁ (2 mg/kg p.o.) and
Table 2. Effect of sodium valproate, piperine and their combination on PTZ induced seizures in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Latency (sec to.)</th>
<th>Frequency of jerks within 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Jerks</td>
<td>Myoclonus and clonic generalised seizures</td>
</tr>
<tr>
<td>A</td>
<td>PTZ</td>
<td>65</td>
<td>333.14±13.468</td>
<td>584.50±60.713</td>
</tr>
<tr>
<td>B</td>
<td>SVP</td>
<td>300</td>
<td>1184.4±275.34**</td>
<td>1475.1±160.58**</td>
</tr>
<tr>
<td>C</td>
<td>P₁</td>
<td>2</td>
<td>502.80±19.873**</td>
<td>905.30±68.250*</td>
</tr>
<tr>
<td>D</td>
<td>P₁</td>
<td>4</td>
<td>661.45±20.083**</td>
<td>931.11±20.028**</td>
</tr>
<tr>
<td>E</td>
<td>SVP+P₁</td>
<td>300+2</td>
<td>1120.6±214.86**</td>
<td>1300.3±170.89**</td>
</tr>
</tbody>
</table>

Values are represented as Mean±SEM. Number of animals: 6; PTZ: Pentylenetetrazole; SVP: Sodium Valproate; P₁: Piperine. Animals not showing seizures in 30 minutes were assigned a latency of 1800 seconds. Dose of PTZ: 65 mg/kg s.c. PTZ given on 5th day of treatment. The vehicle, standard drug and test drugs were given by oral route of administration. Treatment duration: 5 days. * p< 0.05 and ** p< 0.01 versus Group A. Significant by Mann-Whitney test (latencies) and one-way ANOVA followed by Dunnett’s t test (frequency of jerks).

Table 3. Effect of sodium valproate, piperine and their combination in the presence of PTZ on Spontaneous Alternation Behavior in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>% alternation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NS</td>
<td>10 ml/kg</td>
<td>65.236±1.666**</td>
</tr>
<tr>
<td>B</td>
<td>PTZ</td>
<td>65</td>
<td>49.296±3.008</td>
</tr>
<tr>
<td>C</td>
<td>SVP</td>
<td>300</td>
<td>43.026±1.737</td>
</tr>
<tr>
<td>D</td>
<td>P₁</td>
<td>2</td>
<td>60.448±1.437**</td>
</tr>
<tr>
<td>E</td>
<td>P₁</td>
<td>4</td>
<td>68.273±2.306**</td>
</tr>
<tr>
<td>F</td>
<td>SVP+P₁</td>
<td>300+2</td>
<td>70.566±1.059**</td>
</tr>
</tbody>
</table>

Values are represented as Mean±SEM. Number of animals: 6; NS: Normal Saline (0.9% NaCl); PTZ: Pentylenetetrazole; SVP: Sodium Valproate; P₁: Piperine; Dose of PTZ: 65 mg/kg s.c. PTZ given on 5th day of treatment except in Group A. Treatment duration: 5 days. The vehicle, standard drug and test drugs were given by oral route of administration. * p<0.05 and ** p<0.01 versus Group B. Significant by one-way ANOVA followed by Dunnett’s t test.

SVP (300 mg/kg p.o.) significantly increased the % alternation as compared to the toxic control group. Pre-treatment with P₁ (2 and 4 mg/kg p.o.) significantly increased the % alternation. However, % alternation with SVP (300 mg/kg p.o.) was found to be insignificant (Table 3).

Assessment of oxidative stress

**Malondialdehyde estimation**

A significant reduction in the whole brain MDA level by SVP (300 mg/kg p.o.) and piperine (2 and 4 mg/kg p.o.) was observed. The combination of SVP (300 mg/kg p.o.) with piperine (2 mg/kg p.o.) also reduced the MDA levels significantly (Table 4).

**Glutathione estimation**

A significant change in brain GSH level was observed. A significant increase in brain GSH level by SVP (300 mg/kg p.o.) and piperine (2 and 4 mg/kg p.o.) was observed. The combination of SVP (300 mg/kg p.o.) with piperine (2 mg/kg p.o.) significantly increased the GSH levels (Table 5).

DISCUSSION

Epilepsy continues to be a neurological disorder awaiting safer drugs with improved anticonvulsant and anti-epileptogenic effectiveness. Epilepsy is associated with the alternation in psychological, emotional and educational parameters. More than half of the epileptics had some sort of cognitive problems with abnormal behavioral manifestations (Menlove and Reilly, 2015; Motamedi and Meador, 2004). These abnormalities are related to multiple factors including seizure type, age of onset, location of the focus, seizure frequency and the type of EEG pattern (Arif et al., 2009). Another factor that affects cognition is antiepileptic drug therapy (Eddy et
Table 4. Effect of sodium valproate, piperine and their combination in PTZ induced changes of brain MDA levels in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>MDA (nmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NS</td>
<td>10 ml/kg</td>
<td>0.706±0.023**</td>
</tr>
<tr>
<td>B</td>
<td>PTZ</td>
<td>65</td>
<td>0.998±0.074</td>
</tr>
<tr>
<td>C</td>
<td>SVP</td>
<td>300</td>
<td>0.716±0.041**</td>
</tr>
<tr>
<td>D</td>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>2</td>
<td>0.823±0.041*</td>
</tr>
<tr>
<td>E</td>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>4</td>
<td>0.678±0.030**</td>
</tr>
<tr>
<td>F</td>
<td>SVP+P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>300+2</td>
<td>0.618±0.021**</td>
</tr>
</tbody>
</table>

Values are represented as Mean ± SEM. Number of animals: 6; NS: Normal Saline (0.9% NaCl); PTZ: Pentylenetetrazole; SVP: Sodium Valproate; P: Piperine; MDA: Malondialdehyde; Dose of PTZ: 65 mg/kg s.c. PTZ given on 5<sup>th</sup> day of treatment except in Group A. Treatment duration: 5 days. The vehicle, standard drug and test drugs were given by oral route of administration. * p<0.05 and ** p<0.01 versus Group B. Significant by one-way ANOVA followed by Dunnett's t test.

Table 5. Effect of sodium valproate, piperine and their combination in PTZ induced changes of brain GSH levels in mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>GSH (µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NS</td>
<td>10 ml/kg</td>
<td>0.408±0.034**</td>
</tr>
<tr>
<td>B</td>
<td>PTZ</td>
<td>65</td>
<td>0.125±0.024</td>
</tr>
<tr>
<td>C</td>
<td>SVP</td>
<td>300</td>
<td>0.298±0.037*</td>
</tr>
<tr>
<td>D</td>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>2</td>
<td>0.301±0.022*</td>
</tr>
<tr>
<td>E</td>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>4</td>
<td>0.378±0.030**</td>
</tr>
<tr>
<td>F</td>
<td>SVP+P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>300+2</td>
<td>0.395±0.030**</td>
</tr>
</tbody>
</table>

Values are represented as Mean±SEM. Number of animals: 6; NS: Normal Saline (0.9% NaCl); PTZ: Pentylenetetrazole; SVP: Sodium Valproate; P: Piperine; GSH: Glutathione; Dose of PTZ: 65 mg/kg s.c. PTZ given on 5<sup>th</sup> day of treatment except in Group A. Treatment duration: 5 days. The vehicle, standard drug and test drugs were given by oral route of administration. * p<0.05 and ** p<0.01 versus Group B. Significant by one-way ANOVA followed by Dunnett’s t test.

al., 2011; Mula and Trimble, 2009). Although it is understood that the beneficial results of seizure suppression are of great clinical importance, there are indications of cognitive side effects of the drugs, administered at therapeutic doses, especially with polytherapy. Thus, there is a need for drugs which can suppress epileptogenesis and contain cognitive improving property.

Many laboratory models simulate human epilepsy as well as provide a system for studying epileptogenesis (Temkin et al., 2001). In the present study, we used the PTZ model, as it is the most widely employed technique for studying seizure mechanisms and considered to be a useful experimental model for human epilepsy (Mason and Cooper, 1972). SVP was used in the present study since it is broad spectrum, first line drug used in the management of diverse seizure types (Davis et al., 1994; Brodie and French, 2000). It has been categorized as a drug with a narrow margin of safety and with well reported adverse effects on memory. There is need for a drug combination which could bring supra-additive beneficial effects and infra-additive toxicity.

Piperine is reported to exhibit cognition enhancing (Saraogi et al., 2013; Chanpathompikunlert et al., 2010; Wallanthonthorn et al., 2008) and anticonvulsant properties (Bukhari et al., 2013; Chen et al., 2013), so an attempt was made to study its effect per se and in combination of SVP on PTZ induced seizures.

In our study, piperine and SVP per se or in combination attenuated the PTZ induced seizures in mice as evident by the increased latency time and decreased frequency of jerks. These findings are consistent with earlier studies reporting inhibitory effect of piperine on PTZ induced seizures in mice (Bukhari et al., 2013). GABA is the major inhibitory neurotransmitter in the brain, and is widely implicated in epilepsy (Corda et al., 1990). GABAergic neurotransmission enhancement has been shown to attenuate seizures, while inhibition of GABAergic activity facilitates seizures (Okada et al., 1989). The anticonvulsant effect of piperine may be attributed to its effect on GABAergic neurotransmission but the precise mechanism is not known.

Following administration of PTZ we found out increase in oxidative stress as evident by a significant decline in
GSH and an increase in MDA. Pre-treatment with piperine and SVP per se or in combination significantly reduce the PTZ induced oxidative stress as apparent by the increase in GSH and decrease in MDA. Our findings are consistent with the previous studies reporting the antioxidant potential of piperine (Saraogi et al., 2013; Selvendiran et al., 2003). PTZ produced a reduction in % alternation which might be due to its oxidative potential. These results are consistent with the findings of study conducted by Becker et al. (1995), which reported PTZ induced learning impairment in rats. It is well established that SVP causes cognitive impairment (Eddy et al., 2011; Mula and Trimble, 2009; Drane and Meador, 2002). We also observed impairment in SAB following administration of SVP which is seen from the decrease in % alternation. This impairment was successfully reversed when piperine was given in combination with SVP. Piperine has been reported to have cognitive enhancing effect (Saraogi et al., 2013; Chanpathompikunlert et al., 2010; Wallanthorn et al., 2008). Numerous transmitters have been reported to play roles in memory including glutamate, acetylcholine and serotonin (Myhrer, 2003).

Wallanthorn et al. (2008) suggested that the cognitive enhancing effect of piperine probably occur partly via the facilitation of acquisition and consolidation process induced by the alternation in serotonin level. It is also suggested that serotonin interact with acetylcholine to regulate spatial memory (Sirnio et al., 1994; Cassel and Jeltsch, 1995; Steckler and Sahgal, 1995). However the precise mechanism underlying the cognitive enhancing effect of piperine in PTZ model is still not known and requires further investigation.

**Conclusion**

This study presented a preliminary investigation demonstrating that piperine significantly prevented the cognitive impairment and attenuated the oxidative stress induced by the PTZ model. Therefore, it could be useful support to the basic antiepileptic therapy in preventing the development of cognitive impairment reported with several AEDs. However, further studies are required on this drug.

**Conflict of interest**

Authors have none to declare

**REFERENCES**


Full Length Research Paper

Evaluation of metronidazole suspensions

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Most of the formulations of metronidazole suspensions available in circulation tend to have the problem of caking or claying probably due to the flocculation behavior of the dispersed phase. The present study attempts to achieve a desirable flocculation pattern by the use of a polysaccharide, Abelmoschus esculentus (Okra) mucilage alone and then in combination with a monovalent electrolyte and sodium sesquicarbonate (Trona). Metronidazole formulations with various concentrations (1 to 5%w/v) of A. esculentus (Okra), its combination with sodium sesquicarbonate (4:1) and compound tragacanth BP (Reference) were comparatively evaluated using parameters such as sedimentation volume, rheology, pH, redispersibility and particle size. The combined test suspending agents at 5%w/v was found to be superior to the mucilage alone and compound tragacanth BP (P<0.05) at all concentration studied. This result suggests that the combined test suspending agents has the potential of overcoming the problems associated with metronidazole formulations.

Key words: Abelmoschus esculentus (Okra), compound tragacanth BP, metronidazole suspensions, sodium sesquicarbonate (Trona).

INTRODUCTION

A pharmaceutical suspension is a coarse dispersion of insoluble solid particles in a liquid medium. The particle diameter in a suspension is usually greater than 0.5 mm. However, it is difficult and also impractical to impose a sharp boundary between the suspensions and the dispersions having finer particles. Therefore, in many instances, suspensions may have smaller particles than 0.5 mm, and may show some characteristics typical to colloidal dispersions, such as Brownian movement (Martin, 2006). A suspending agent is a substance that increases the viscosity of a suspension, so that sedimentation is retarded. The presence of a suspending agent is required to overcome agglomeration of the dispersed particles and to increase the viscosity of the medium, so that the particles may settle slowly. These suspending agents increase sedimentation volume, ease redispersibility, enhance pour ability and prevent compact formation. Suspending agents can be grouped into three classes: Synthetic; semi-synthetic and the natural polysaccharide, which, tragacanth, okra and acacia...
belong.

Gum of the *Abelmoschus esculentus* (okra) family (Malvaceae) is a natural polymer consisting of D-galactose, L-rhamnose and L-galacturonic acid (Agarwal et al., 2001).

Gums of *A. esculentus* (Okra) pods have been reported to have binder potential for tablet formulations (Ofoefule et al., 2001; Chopra et al., 1956). The fresh fruits of *A. esculentus* (L.) are a common component of Indian diet. In addition, the plant has been used medicinally in treatment of several disorders (Chopra et al., 1956; Jha et al., 1997). Anti-cancer, antimicrobial and hypoglycemic activities of the plant are reported (Pal et al., 1968; Tomoda et al., 1987). The anti-ulcer activity of fresh fruits is recently reported (Gurbuz et al., 2003). Studies have also been carried out for its use as a suspending agent (Kumar et al., 2009). Trona is an evaporite mineral also known as sodium sesquicarbonate. A white crystalline hydrated double salt, Na$_2$CO$_3$·NaHCO$_3$·2H$_2$O, soluble in water but less alkaline than sodium carbonate and decomposes on heating. Metronidazole a nitrimidazole antibiotic medication is used particularly for anaerobic bacteria and protozoa. It is antibacterial against anaerobic organisms, an amoebicide, and an antiprotozoal (Cohen et al., 2010). It is the drug of choice for first episodes of mild-to-moderate clostridium difficile infection (Cohen et al., 2010).

This study aims to investigate the suspending properties of the gum, and its combination with sodium sesquicarbonate (Trona) on the stability of metronidazole suspension.

**MATERIALS AND METHODS**

Metronidazole powder (Medex co. ltd), benzoic acid solution B.P., amaranth solution (E- Merck Darmstardt Germany), raspberry syrup, chloroform water- double strength (BDH Laboratory Supplies Poole, England), compound tragacanth powder (BDH Laboratory Supplies Poole, England) and ethanol 96% (BDH Laboratory Supplies Poole, England) were used for this study. *A. esculentus* (okra) fruits and trona (Sodium sesquicarbonate) were purchased from the local market in Sagamu, Ogun State, Nigeria and demineralized water was gotten from Faculty of Pharmacy, Olabisi Onabajo University, Ogun State, Nigeria.

**Extraction of the gum from the fruit of *A. esculentus* (Family - Malvaceae)**

The fruits were sun dried for three weeks and were size reduced using pestle and mortar into powder. The powder was then sifted using a sieve size of 250 μm. Four hundred grams (400 g) of dried powdered okra was dispersed in 8 L of demineralized water and kept in refrigerator for 24 h. The dispersed mucilage was strained through a muslin cloth to remove the fibrous materials. The cloudy mucilage was clarified by centrifugation at 15,500 revolutiohs per minute using centrifuge (Model LF-400R, Biomaker, China). The clarified mucilage was transferred into a 1 L beaker and the gum was extracted with 8 L of ethanol 96%. The extracted gum was redispersed in water and re-extracted with 4 L of ethanol 96% to get a whitish gum. The gum was dried in an oven at 65°C for 8 h. The dried gum obtained was milled into powder.

**Purification of trona (sodium sesquicarbonate)**

Na$_2$CO$_3$·NaHCO$_3$·2H$_2$O

Trona was obtained from a local market in Sagamu, Ogun state, was size reduced in a mortar and pestle. The powder was then sifted using a sieve size of 250 μm. 100 g of the powdered trona was dispersed in 1L of demineralised water. This was passed through a muslin cloth with the residue removed. The cloudy filtrate was allowed to settle for 30 min, after which the supernatant fluid was decanted. The sediment was dried in an oven at 80°C for 3h. The dried flakes obtained were then milled into powder using a blender.

**Preparation of metronidazole suspension**

5 g of metronidazole powder and 1g of compound tragacanth powder were weighed and triturated in a mortar. Twenty ml of raspberry syrup was added in aliquot proportion and triturated for 3 min until a smooth paste was formed. 2 ml of benzoic acid and 1 ml of amaranth solutions were added gradually with constant stirring and then mixed with 50 ml of chloroform water double strength. The mixture was transferred into a 200 ml amber bottle made up to 100 ml volume with distilled water and then shaken vigorously for 2 min (thus making 1%(w/v) of the gum in the preparation). The procedure was repeated using different concentrations 2, 3, 4 and 5% (w/v) of compound tragacanth gum. The method above was used for okra gum and combination of okra gum plus trona (4:1) except that in case of okra gum, the appropriate amount was weighed and soaked in 2 ml of demineralized water and allowed to soak for 12 h while kept in the refrigerator before triturating with metronidazole powder. Also, the mixture of okra gum and trona (4:1) was first weighed into an evaporating dish and sufficient water was added to form watery mucilage before being heated on a water bath at 100°C for 1 min till viscous mucilage was formed. This was allowed to cool, and then triturated as appropriate with metronidazole powder before other ingredients were incorporated (Table 1).

**Determination of sedimentation volume**

20 ml of each suspension was stored in 30 ml cylinder in replicate and was left undisturbed. The sedimentation volume of the suspension was then determined at room temperature for 45 days, the result was taken at every 5 days. The sedimentation volume was observed and noted. The sedimentation volume is denoted by (F)

\[
F = \frac{V_u}{V_o} \quad (1)
\]

Where $V_u$ is the ultimate volume of the sediment and $V_o$ is the original volume of the suspension.

**Rheological assessment**

The time to empty 5 ml of the suspension in duplicate
from a 5 ml pipette was calculated to give the flow rate and computed thus;

\[ \text{Flow rate} = \frac{\text{Volume of pipette (ml)}}{\text{Flow time (s)}} \]  

Likewise, the shear rate was also calculated

\[ \text{Shear rate} = \frac{\text{Flow rate (ml/s)}}{\text{Volume of suspension}} \]

The viscosity behavior of the suspensions prepared with compound tragacanth gum, A. esculentus (Okra) gum and a combination of A. esculentus (Okra) and sodium sesquicarbonate (Trona) in duplicate were studied using shanghai changji rotational viscometer, spindle number 2 and 3 of low viscosity type with gear speed ranging 30 to 60 rpm. Using these observations, the rate of shear was calculated. The results were recorded as shown in Table 4.

**Particle size analysis**

The particle size distribution of metronidazole powder was determined micro metrically (Martin et al., 1993). The sieves in descending order of aperture size were arranged from 1000 to 90 µm mesh size and together with collection pan (receiver) was shaken using a sieve shaker. 10 g of metronidazole powder was weighed into the sieve with the largest aperture. After shaking for 5 min, the weight of the particle retained on each sieve was determined and recorded appropriately. The mean particle diameter was determined using the formula:

\[ \text{Mean diameter} = \frac{\sum(\% \text{ weight retained } \times \text{ mean aperture})}{100} \]  

**RESULTS AND DISCUSSIONS**

The average yield of dried gum obtained from A. esculentus fruit was found to be 16.75 % (w/w). The gum powder obtained was a light brown powder with no taste or odours.

**Sedimentation volume**

Sedimentation volume is the ratio of the height of the sediment after settling and initial height of suspension. The larger the ratio, the better is the suspendability (Patel et al., 1976). The average sedimentation volume measured for the formulations at different concentration of suspending agents are shown in Table 2 and Figure 1 to 5. The result shows that all the samples settled to form sediments at one time or the other. This is probably due to interaction between the suspended particles which eventually led to generation of energy. This is in conformity with observations of Tabibi and Rhodes (1995) who defined energy of interaction \( V_T \) between two particles as

\[ V_T = V_R + V_A \]

Where \( V_T \) = Total energy of interaction, \( V_R = \) Repulsive forces, \( V_A = \) Attractive forces. When \( V_R > V_A \), there is deflocculation and when \( V_A > V_R \), there is flocculation which in either case will eventually lead to sedimentation. Suspension sample produced using okra and the combination of both okra and trona showed a higher sedimentation volume when compared with those prepared using compound tragacanth at all concentrations of prepared samples. Suspension samples containing the combination of okra and trona at concentrations 1, 2, 3, and 4%(w/v), respectively showed only a slight increase in sedimentation volume when compared with those of corresponding concentrations of okra with the exception of those containing 5%(w/v) of the combination of okra and trona, where there is a higher increase in sedimentation volume. Generally, the sedimentation volume of all the formulations were of the order Okra + trona > Okra > Compound Tragacanth
Table 1. Metronidazole suspension formulation.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Batch 1 (%w/v)</th>
<th>Batch 2 (%w/v)</th>
<th>Batch 3 (%w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Metronidazole powder (g)</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Compound tragacanth (g)</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Okra gum powder (g)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trona (g)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amaranth solution (ml)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Benzoic acid solution (ml)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Raspberry syrup (ml)</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Chloroform water double strength (ml)</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Water for preparation q.s</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Effect of varying concentration of suspending agents on the sedimentation volume of Metronidazole suspension.

<table>
<thead>
<tr>
<th>Suspending agents</th>
<th>Concentration of suspending agent (%w/v)</th>
<th>Mean sedimentation volume (cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Time (days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Compound tragacanth gum</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.00</td>
</tr>
<tr>
<td>Okra gum</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.00</td>
</tr>
<tr>
<td>Okra and trona in ratio</td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>4:1</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.00</td>
</tr>
</tbody>
</table>

(P<0.05). This observation could be explained thus, sodium sesquicarbonate (Trona) being acted as flocculating agent by reducing the electrical barrier between the particles, thereby leading to formation of a bridge between adjacent particles linking them together in a loose arranged structure. Okra in the combination formulation however, may be acting in retarding the sedimentation of the floccules so formed by the effect of sodium sesquicarbonate (Trona) behaving as a hydrocolloid suspending agent. As can be observed from Table 2 and Figure 1 to 5 as concentration of the suspending agents increased, the ability to retard sedimentation of floccules increased.

Rheological assessment

The average flow rate of different concentrations of suspension formulation from where shear rate was calculated using equation 3 is presented in Table 3. The
Table 3. Effects of varying concentration of suspending agents on the flow rate (ml/sec) and shear rate of Metronidazole suspension.

<table>
<thead>
<tr>
<th>Suspending agents</th>
<th>Concentration of suspending agent (%w/v)</th>
<th>Mean flow rate (ml/sec)</th>
<th>Shear rate (sec^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>1</td>
<td>0.185±0.0001</td>
<td>0.0370</td>
</tr>
<tr>
<td>Tragacanth gum</td>
<td>2</td>
<td>0.035±0.002</td>
<td>0.0070</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.023±0.001</td>
<td>0.0046</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.015±0.001</td>
<td>0.0030</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.013±0.001</td>
<td>0.0026</td>
</tr>
<tr>
<td>Okra gum</td>
<td>1</td>
<td>0.100±0.001</td>
<td>0.0200</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.035±0.002</td>
<td>0.0070</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.026±0.001</td>
<td>0.0050</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.019±0.001</td>
<td>0.0040</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Okra and trona in ratio 4:1</td>
<td>1</td>
<td>0.208±0.001</td>
<td>0.0420</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.047±0.001</td>
<td>0.0090</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.027±0.001</td>
<td>0.0050</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

viscosity of these formulations using spindle 2 and 3 are shown in Table 4. Generally, for all formulations, as the flow rate was decreasing along with the increase in concentration of suspending agents, the viscosity was increasing as shown in Tables 3 and 4. For the formulation 5% (w/v) okra gum as suspending agent,
Table 4. Effect of the type and concentration of suspending agent on the viscosity of Metronidazole suspension.

<table>
<thead>
<tr>
<th>Suspendin(\text{g}^\text{ing agents}</th>
<th>\text{Concentration of suspending agent (%w/v)}</th>
<th>\text{Mean viscosity (Poise)}</th>
<th>\text{Spindle speed (revolutions per min)}</th>
<th>\text{Spindle speed (revolutions per min)}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spindle 2</td>
<td>Spindle 3</td>
<td>30</td>
</tr>
<tr>
<td>Compound tragacanth gum</td>
<td>1</td>
<td>0.38±0.03</td>
<td>0.30±0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.00±0.15</td>
<td>1.86±0.03</td>
<td>2.05±0.23</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.33±0.25</td>
<td>3.06±0.34</td>
<td>3.00±0.29</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.15±0.15</td>
<td>3.66±0.27</td>
<td>4.04±0.56</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.49±0.23</td>
<td>4.17±0.56</td>
<td>4.17±0.42</td>
</tr>
<tr>
<td>Okra gum</td>
<td>1</td>
<td>1.39±0.01</td>
<td>1.13±0.01</td>
<td>1.33±0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.89±0.62</td>
<td>3.66±0.12</td>
<td>4.68±0.34</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.46±0.45</td>
<td>4.07±0.23</td>
<td>5.47±0.23</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>12.06±0.68</td>
<td>8.42±0.34</td>
<td>10.36±0.42</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20.62±1.12</td>
<td>11.59±0.56</td>
<td>18.38±0.73</td>
</tr>
<tr>
<td>Combination okra and trona 4:1</td>
<td>1</td>
<td>0.52±0.01</td>
<td>0.43±0.01</td>
<td>0.86±0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.39±0.16</td>
<td>2.56±0.17</td>
<td>3.56±0.72</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.31±0.97</td>
<td>5.17±0.33</td>
<td>7.51±0.93</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9.50±0.98</td>
<td>6.75±0.65</td>
<td>9.48±0.94</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>21.64±1.15</td>
<td>11.61±0.71</td>
<td>16.58±0.99</td>
</tr>
</tbody>
</table>

Figure 2. Comparism of sedimentation volume among suspension formulations containing 2% w/v concentration agents.

4%\(\text{w/v}\) and 5%\(\text{w/v}\) respectively for combination of okra gum and Trona (4:1), the viscosities were very high at 30 and 60 revolutions per minutes using spindle 2 and 3 as shown in Table 4. This is so much that the flow rate and shear rate at the stated concentrations above gave zero value as as shown in Table 3. The possible reason
Figure 3. Comparism of sedimentation volume among suspension formulations containing 3% w/v concentrations of suspending agents.

Figure 4. Comparism of sedimentation volume among suspension formulations containing 4% w/v concentrations of suspending agents.
for this observed occurrence is that the degree of flocculation can impede the rheological property of a pharmaceutical formulation. This is because the amount of free continuous phase is reduced as more flocules are formed. The viscosity of the combination of Okra gum and Trona (4:1) at 5% (w/v) was the highest throughout as shown in Table 4. Again the degree of flocculation is possibly responsible for this since it contains an electrolyte flocculating agent as has been explained earlier on. When a disperse system is highly flocculated, the possibility of interactions between flocules increases and structured systems result (Aulton, 1996). If the forces bonding flocules together are capable of withstanding weak stresses, then a yield value will result and below this value, the suspension will behave like a solid that will be difficult to flow, hence the zero flow rate observed for Figure 5. Comparism of sedimentation volume among suspension formulations containing 5% w/v concentrations of suspending agents.

Table 5. Particle size distribution of metronidazole powder.

<table>
<thead>
<tr>
<th>Particle size (µM)</th>
<th>Weight retained (G)</th>
<th>Percentage weight retained (%)</th>
<th>Cumulative percentage frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;1000</td>
<td>1.8</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>&gt;710</td>
<td>2.1</td>
<td>1.05</td>
<td>1.95</td>
</tr>
<tr>
<td>&gt;500</td>
<td>4</td>
<td>2</td>
<td>3.95</td>
</tr>
<tr>
<td>&gt;355</td>
<td>7</td>
<td>3.5</td>
<td>7.45</td>
</tr>
<tr>
<td>&gt;250</td>
<td>96.9</td>
<td>48.45</td>
<td>55.9</td>
</tr>
<tr>
<td>&gt;150</td>
<td>39</td>
<td>19.5</td>
<td>75.4</td>
</tr>
<tr>
<td>&gt;90</td>
<td>30.3</td>
<td>15.15</td>
<td>90.55</td>
</tr>
<tr>
<td>Receiver</td>
<td>18.8</td>
<td>9.45</td>
<td>100</td>
</tr>
</tbody>
</table>

Mean Particle diameter = 379.77µm.
Table 6. The pH of Metronidazole suspension using varying concentration of suspending agent.

<table>
<thead>
<tr>
<th>Suspending agents</th>
<th>Concentration of suspending agent (%w/v)</th>
<th>Mean pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Time (days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Compound tragacanth</td>
<td>1</td>
<td>8.7±0.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9.0±0.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9.3±0.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9.2±0.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>9.3±0.3</td>
</tr>
<tr>
<td>Okra gum</td>
<td>1</td>
<td>8.6±0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.7±0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.7±0.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.7±0.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8.9±0.3</td>
</tr>
<tr>
<td>Okra and trona in ratio 4:1</td>
<td>1</td>
<td>9.3±0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9.2±0.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10.1±0.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10.3±0.3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10.1±0.3</td>
</tr>
</tbody>
</table>

this concentration 5%(w/v).

**Particle size analysis**

The mean particle diameter and the particle size distributions of suspended insoluble drugs are important considerations in formulating physically stable pharmaceutical suspensions. Drug particle size is an important factor influencing product appearance, settling rates, drug solubility, in vivo absorption, resuspendability and overall stability of pharmaceutical suspensions (Nash, 1966). According to stoke's equation,

\[
\nu = \frac{d^2 (\rho_1 - \rho_2) g}{18 \eta} = \frac{2 r^2 (\rho_1 - \rho_2) g}{9 \eta}
\]

where \( \nu \) is the velocity of sedimentation; \( d \) and \( r \) are the diameter and radius of the particle respectively; \( \rho_1 \) and \( \rho_2 \) are the densities of the dispersed phase and dispersion medium; \( g \) is the acceleration due to gravity; and \( \eta \) is the viscosity of the dispersion medium.

The velocity of sedimentation is directly proportional to the square of the diameter of the suspended particle, this implies that the larger the diameter of the suspended particles the faster such particle sediment thereby, leading to shorter suspending time and ultimately caking, the smaller the particle diameter the slower the sedimentation rate and the longer the suspending time, these results in better suspension as redispersibility is easy and accurate dosage can be withdrawn. The particle size distribution data of metronidazole powder is presented in Table 5, and the size analysis is shown in Figure 6. The figure represents a unimodal frequency distribution. The particle size is in the range of 90 to 1000 µm. The mean diameter for the particle of the powder was calculated and was found to be 379.77 µm. The relatively large size of the suspended particle may be the reason for its initial high rate of sedimentation observed for the first five days of preparation until the terminal settling velocity was reached after which the settling rate remained steady. From Figure 6, it was observed that 7.5% of the metronidazole particles were greater than 250 µm, 68.3% were less than 250 µm and 24.7% were less than 90 µm. This shows that the bulk of the
size of the particle is less than 250 µm but greater than 90 µm resulting in most of the particles in the formulation being suspended over a longer period of time which reduces greatly the tendency of such formulation to cake, and therefore improves its suspending character.

**pH**

The pH of all formulations is presented in Table 6. It could be observed that in the first 20 days of the study, pH appears to be in the alkaline to neutral region, as the storage progressed up to 40 days there appear to be a drop in the values of the pH of all formulations tending towards neutral to acidic zone. The change in pH may be a signal to microbial degradation of the polymer suspending agent into constituents like D-galactose, L-rhamnose and L-galactouronic acid. It was also noted that tests formulations containing A. esculentus (Okra) and sodium sesquicarbonate (Trona) were found to have the highest pH values throughout the period of study. This is likely to be due to the presence of sodium sesquicarbonate (Trona) that in itself is basic in nature.

**Redispersibility**

Suspensions tend to settle with time, and therefore, they are designed to be readily redispersed by gentle shaking or stirring, resulting in a homogeneous suspension. Redispersability depends on particle size of the dispersed phase and the nature of suspension formed (floccules, non-floccules or coagule). Since the suspensions produced sediment on storage, it must readily be dispersible so as to ensure the uniformity of the dose. The redispersing ability of the suspandants was shown in Table 7. It was observed that for suspensions prepared using compound tragacanth, the degree of redispersibility reduces as the concentration of the suspending agent increases, inferring that the higher the concentration of the suspending agent the more difficult it would be to redisperse, if the sediments form cake, this was not the case for suspensions produced using okra and those produced using the combination of okra and trona, which showed an increase in the degree of redispersibility as their concentrations increases, inferring that the degree of redispersibility is directly proportional to the concentration of suspending agent used. The observed contrast in the redispersing ability of tragacanth formulated suspension and those formulated using okra and the combination of okra and trona may be attributed to the nature of the suspension formed, since suspensions containing tragacanth formed a deflocculated suspension, they tend to form sediments that eventually cake over time, which are difficult to redisperse, while on the other hand, suspensions
Table 7: Redispersibility of suspensions after shaking 30 times over a period of 30 days.

<table>
<thead>
<tr>
<th>Suspending agents</th>
<th>Concentration of suspending agent (%w/v)</th>
<th>Redispersibility time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Compound tragacanth gum</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Okra gum</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Combination of okra and trona in ratio 4:1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

Key: +++ = re-dispersible with vigorous agitation and stable enough for adequate dose withdrawal, ++ = easily re-dispersed with minimum agitation and stable enough for adequate dose withdrawal, -- = Not re-dispersible, formed hard cake.

containing okra and the combination of okra and trona produced a flocculated suspension, which on settling form loose flocs that are easily redispersed on shaking (Lucks et al., 1990). It was also observed that degree of redispersibility decreases as storage time increases for all formulations.

Conclusion

The stability of metronidazole suspension was improved by the addition of the combination of okra and trona in the ratio 4:1, and those formulated with okra alone. The physicochemical properties of the formulations were evaluated. The results showed that the sedimentation volume and viscosity were directly proportional to the concentration of the suspending agents. The reverse case was observed with the flow rate. The present study reveals that metronidazole suspension formulated with the combination of okra and trona and those containing okra alone as suspending agents have better stability indicated by enhancing suspending properties when compared to the conventional compound tragacanth. On the other hand, metronidazole suspension formulated with the combination of okra and trona showed an observable improvement in its suspending properties especially at higher concentrations of okra, inferring that addition of trona improves the suspending property of okra gum. It can therefore, be concluded that addition of trona in concentration ratio of 1:4 to okra gum as suspending agent, influences and impacts a better stability and enhancement of physicochemical properties on the metronidazole suspension especially at higher concentration of okra gum. Therefore, the combination of locally source okra and trona has potential to be used as suspending agent especially in suspensions experiencing caking problem as a result of sedimentation such as metronidazole suspension.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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