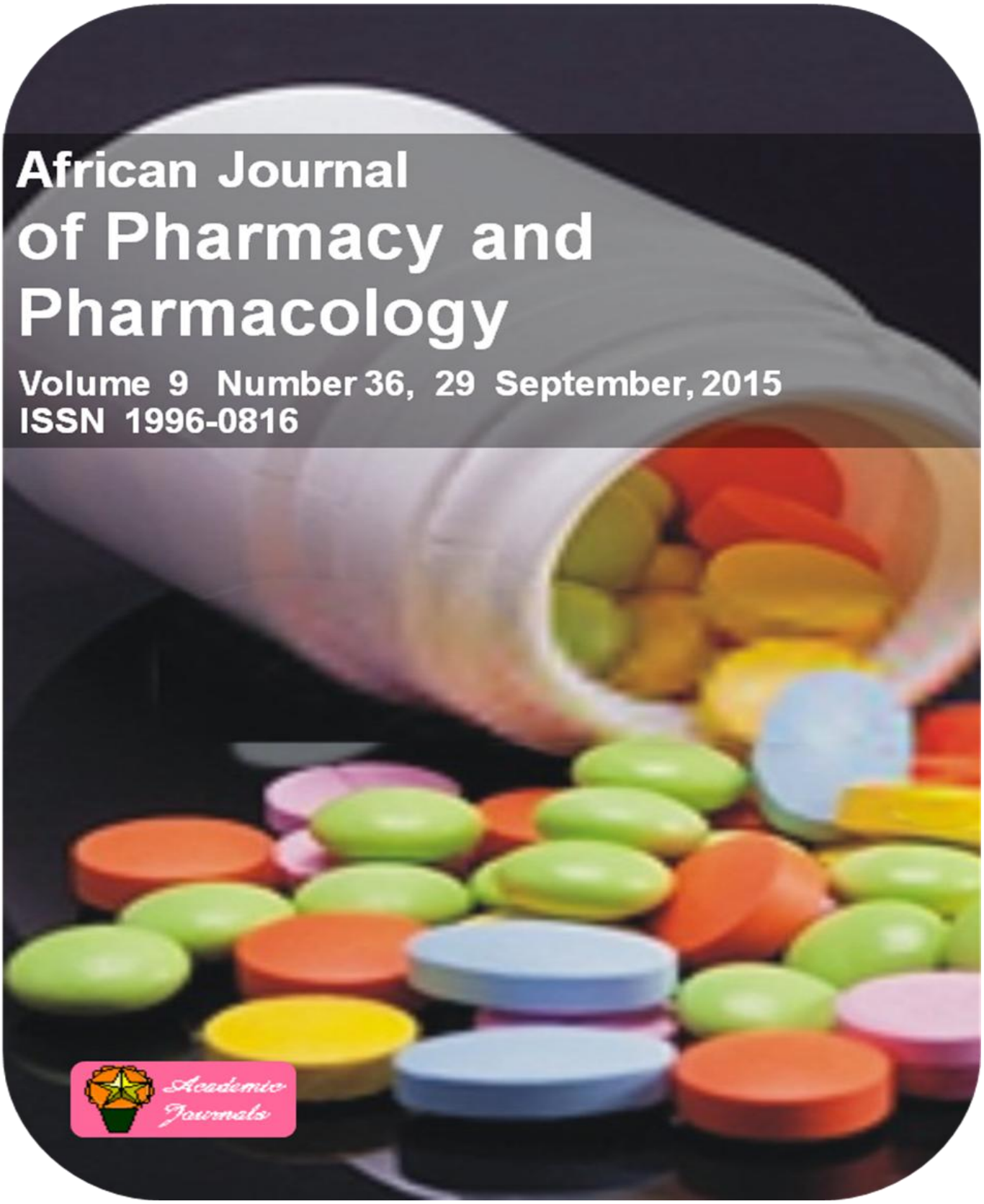

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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Anti-nociceptive and anti-inflammatory activities of *Holoptelea intrigofolia* (Roxb.) planch fruit extract on laboratory animals

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The methanolic extract of the fruit of *Holoptelea intrigofolia* (Roxb) Planch was investigated for its anti-inflammatory and analgesic activities in animal models. The extract at 100, 150 and 200 mg/kg body weight reduced significantly the formation of edema induced by carrageenan. In the acetic acid-induced writhing model, the extract showed a good analgesic effect characterized by reduction in the number of writhes when compared to the control. The analgesic effect of extract of *H. intrigofolia* was also significant ($P < 0.05$) in tail immersion test and was dose dependent. These results were also comparable to those of diclofenac sodium (50 mg/kg), the reference drugs used in this study. Though the study has provided some justification for the folkloric use of the plant in several communities for conditions such as pain and inflammations but caution should be exercised in its use for medicinal purpose.

Key words: *Holoptelea intrigofolia,* anti-nociceptive activity, tail flick test, hot plate test, anti-inflammatory activity.

INTRODUCTION

The world has changed so much so has the need of human beings changed for the treatment of different disease. Researchers are trying to find out alternative medicines through natural sources; there are so many plants that have potential medicinal properties which have to be discovered, and this process of exploring the natural sources must go on. Herbal medicines are cost effective and have fewer side effects (Samsam and Moatar, 1991). According to World Health Organization (WHO), 80% of the people worldwide rely on herbal medicines for some aspects. It is estimated that there are 250,000 to 500, 000 species of plants on earth, out of which 75,000 plants species are of medicinal value and only 800 plants species are used in the preparation of herbal drugs, so there is comprehensive scope of finding new molecule (Borris, 1996).

In continuation of this exploring of the natural sources, the plant *Holoptelea intrigofolia* (Roxb) Planch plays a vital role. *H. intrigofolia* (Ulmaceae) is distributed over tropical and temperate regions on Northern hemisphere

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including Pakistan, India peninsula to indo china, and Srilanka. The common vernacular names of the plant are Papri, Magzi (Urdu), Chilbil, Dhamma, Begana (Hindi) (Mahmud et al., 2010; Anonymous, 2001). It is traditionally used in the treatment and prevention of several ailments like leprosy, inflammation, rickets, leucoderma, scabies, rheumatism, ringworm, eczema, malaria, intestinal cancer, and chronic wounds. *In vitro* and *in vivo* pharmacological investigations on crude extracts and isolated compounds showed antibacterial, antifungal (Vinod et al., 2010a, b), antioxidant (Saraswathy et al., 2008), anti-inflammatory (Srinivas et al., 2009), anthelmintic (Durga and Paarakh, 2010), antidiabetic (Sharma et al., 2010), antidiarrhoeal (Sharma and Lakshami, 2009), adaptogenic (Puri et al., 2011), anticancer (Lakshmi et al., 2010), wound healing (Srinivas et al., 2008a), hepatoprotective (Hemamalini and Sathya, 2013), larvicidal (Singha et al., 2012), antiemetic (Srinivas et al., 2008b) and central nervous system (CNS) depressant (Hemamalini et al., 2011) and analgesic activities (Rizwani et al., 2012). Phytochemical analysis showed the presence of terpenoids, sterols, saponins, tannins, proteins, carbohydrates, alkaloids, phenols, flavonoids, glycosides, and quinines. Numerous compounds including holoptelin-A, holoptelin-B, friedlin, epifriedlin, β-amyrin, stigmasterol, β-sitosterol, 1, 4-napthalenedione, betulin, betulinic acid, hexacosanol, and octacosanol have been identified and isolated from the plant species (Harleen et al., 2011).

The basic aim of our study is to determine the anti-nociceptive and anti-inflammatory activity of *H. intrigofolia* fruit extract which is not explored yet.

MATERIALS AND METHODS

Collection and extract preparation

The fresh fruit of *H. intrigofolia* was collected from the premises of University of Karachi. The plant was taken to the harbium of Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi where the plant was identified by Prof. Dr. Ghazala H. Riwani. It was dried under shade and macerated in methanol at room temperature for 15 days. This was then filtrated using filter paper (Whatmann size no. 1) and filterate was evaporated using rotary evaporator (Buchi, Switzerland). The dried residue of fruit extract of *H. intrigofolia* was kept in air tight bottle until it was reconstituted for administration.

Management of experimental animals

Strain of albino Wister rats (150 to 160 g) and mice (20 to 25 g) of both sexes were procured from the Animal House of Department of Pharmacology, Dow University of Health Sciences, Pakistan. The animals were kept in well aerated laboratory cages in the animal house of Department of Pharmacology, Faculty of Pharmacy, University of Karachi. They were allowed to acclimatize to the laboratory environment for a period of one week before the commencement of the experiment. The animals were given access to standard feed and drinking water during the acclimatization period.

Acetic acid induced writhing

Acetic acid was used for assay of analgesic potential of extract of *H. intrigofolia*. Mice weighing 25 g were used for the activity. Group I received normal saline while Group II standard drug diclofenac sodium 50 mg/kg orally. While the remaining groups III, IV and V were orally administered with 100, 150 and 200 mg/kg of the extract of *H. intrigofolia,* respectively. After 30 min of drug administration, pain was induced by intraperitonial administration of 0.6% acetic acid. The number of abdominal constrictions (writhes) was counted after 5 min of acetic acid injection for the period of 10 min (Koster et al., 1959).

Hot plate test

For hot plate analgesic activity, mice were placed on a hot plate (50 ± 2°C) and their reaction to heat was observed. Five groups of 6 mice each were used. Group I received normal saline while Group II standard drug diclofenac sodium 50 mg/kg orally. While the remaining groups III, IV and V were orally administered with 100, 150 and 200 mg/kg of the extract of *H. intrigofolia,* respectively. When the animal raised and licked the front paws they were quickly removed from the hot plate and the time period observed. The observations were recorded after 30 min of drug administration (Dharmasiri et al., 2003). The cut-off time, that is time of no response was put at 30 s. Percent analgesia was calculated using the following formula:

% Analgesia = (Test latency – control latency) / (Cut – off time – control latency) × 100

Tail flick test

Tail flick method was used for analgesic activity. Mice were divided into four groups of five animals each. Group I received normal saline while Group II standard drug diclofenac sodium 50 mg/kg orally. While the remaining groups III, IV and V were orally administered with 100, 150 and 200 mg/kg of the extract of *H. intrigofolia*, respectively. In this method, tail was immersed in water heated at 50 ± 2°C in water bath. The time period in which tail flicked out from water bath was recorded. The observations were made for 180 min at every 30 min interval (Owoyele et al., 2004). The reaction time was determined at 30, 60, 90, 120,150 and 180 min after treatment. Baseline was considered as reaction time before administration of extracts or reference drug. The cut-off time was taken as 10 s to prevent tissue damage. Tail flick antinociceptive index (TFAI) was calculated from the expression:

TFAI = reaction time-baseline/cut-off baseline

Carrageenan-induced rat paw edema

Thirty rats were used in this study and they were divided into five groups of six per group. Group I received normal saline while Group II standard drug diclofenac sodium 50 mg/kg orally. While the remaining groups III, IV and V were orally administered with 100, 150 and 200 mg/kg of the extract of *H. intrigofolia*, respectively. Acute inflammation was produced by the sub-plantar administration of 0.1 ml of 1% carrageenan in normal saline in the right paw of rats. The paw volume was measured at 0, 1, 2, 3, 4 and 5 h after carrageenan injection using a micrometer screw gauge. Increases in the linear diameter of the right hind paws were taken as an indication of paw edema. The percentage inhibition of the inflammation was calculated from the formula:

Table 1. Effect of extract of *H. integrifolia* on acetic acid induced writhing in mice.

Treatment	Dose (mg/kg)	Mean No. of writhes±S.E.M
Control	-	55±1.86
Diclofenac sodium	50	16±1.382**
Extract of *H. integrifolia*	100	38.80±2.50**
	150	28.00±1.50**
	200	20.00±1.14**

Values are expressed as mean ± S.E.M (n=6). Statistical significance were calculated by ANOVA followed by Tukey post hoc test when compare to the control group *P < 0.05, ** P< 0.01

Table 2. Effect of extract of *H. integrifolia* on hot plate method.

Treatment group	Dose (mg/kg)	Reaction time						
		0 min	30 min	60 min	90 min	120 min	150 min	180 min
Control	-	5.17±0.04	5.24±0.03	6.39±0.14	5.55±0.14	6.60±0.02	5.88±0.2	5.98±0.25
Diclofenac sodium	50	8.33±0.6	10.10±0.7	12.34±0.7	13.7±0.7	14.45±1.1	11.54±0.7	10.23±0.8
Extract of *H. integrifolia*	100	8.81±0.7	16.40±3.7**	13.40±4.7	9.20±4.0	7.00±3.3	7.60±3.2	12.40±4.7
	150	9.20±0.8	21.80±7.5**	20.60±12.1*	14.20±8.8	21.20±14.5*	14.4±10.3	15.20±4.7
	200	7.80±0.8	13.80±4.4*	13.20±5.6	11.20±4.0	9.60±4.0	12.0±6.5	12.40±5.31

Values are expressed as mean ± S.E.M (n=6). Statistical significance were calculated by ANOVA followed by Tukey post hoc test when compare to the control group *P < 0.05, ** P< 0.01

% Inhibition of inflammation = D0 – Dt / D0 × 100

Where D0 was the average inflammation (hind paw edema) of the control group of rats at a given time; and Dt was the average inflammation of the drug treated (that is, extracts or reference diclofenac sodium) rats at the same time (Sawadogo et al., 2006; Moody et al., 2006).

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). The data obtained were statistically analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison post hoc test to determine the level of significance between control and experimental group. Values of P < 0.05 were considered as significant.

RESULTS

Acetic acid-induced writhes

Peripheral analgesic activity of extract of *H. intrigofolia* was assessed by acetic acid induced writhing method. The results showed that the pain relief was achieved in a dose dependent manner, at all test doses (100, 200 and 300 mg/kg, o.p.) when compared to the diclofenac sodium (50 mg/kg) as shown in Table 1.

Hot plate test

The results of the hot plat test revealed that the latency time was significantly (P < 0.05) increased from 8.81 ± 0.7 to 12.40 ± 4.7 at the dose of 100 mg/kg after 180 min as shown in Table 2. The most significant (P < 0.01) increase in latency time noticed against 150 mg/kg of extract of *H. intrigofolia* was 21.80 ± 7.5 whereas, the percent analgesia of the standard diclofenac sodium and extract of *H. intrigofolia* (HIE) shown in Figure 1.

Tail flick Test

The analgesic effect of extract of *H. intrigofolia* was also significant (P < 0.05) in tail immersion test and was dose dependent. The reaction time of all doses and diclofenac sodium is given in Table 3. The maximum analgesic effect was noticed at 60 min after the dose administration. Tail flick antinociceptive index (TFAI) of *H. intrigofolia* (HIE) at 100, 150 and 200 mg/kg, respectively is presented in Figure 2.

Carrageenan-induced rat paw edema

The anti-inflammatory activity at test doses (100, 150 and

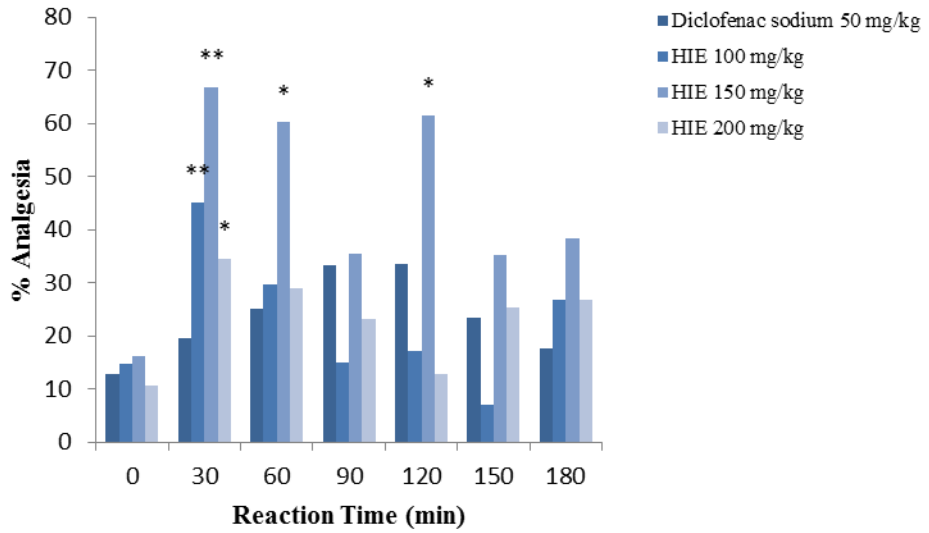

Figure 1. Percent effect of extract of *H. integrifolia* (100, 150 and 200mg/kg) and diclofenac sodium (50mg/kg) on hot plat pain model in mice. Each point represent the mean ± SEM of six animals. The data was analyzed by ANOVA followed by Tukey's test. Asterisks indicated statistically significant values from control. *P < 0.05, **P < 0.01.

Table 3. Effect of extract of *H. integrifolia* on Tail- flick response in mice

Treatment group	Dose (mg/kg)	Reaction time						
		0 min	30 min	60 min	90 min	120 min	150 min	180 min
Control	-	1.30±0.39	1.03±0.17	0.99±.008	1.10±1.4	1.03±0.4	1.03±0.4	1.01±0.4
Diclofenac sodium	50	1.33±0.2	3.25±0.2*	5.59±0.71**	6.55±0.54**	6.18±0.6**	5.6±0.53**	4.49±0.41**
	100	1.37±0.47	3.87±0.84**	6.30±0.78*	6.54±1.18**	5.90±2.1**	5.45±1.1**	2.75±1.1**
Extract of *H. integrifolia*	150	1.32±0.21	4.23±1.49**	7.58±5.0**	9.18±3.5**	6.70±1.7**	4.73±1.0**	3.24±0.6*
	200	1.38±0.19	4.75±1.6**	9.86±3.2**	9.13±1.2**	8.40±1.3**	6.98±0.5**	5.02±0.9**

Values are expressed as mean ± S.E.M (n=6). Statistical significance were calculated by ANOVA followed by Tukey's post hoc test when compare to the control group *P < 0.05, **P< 0.01

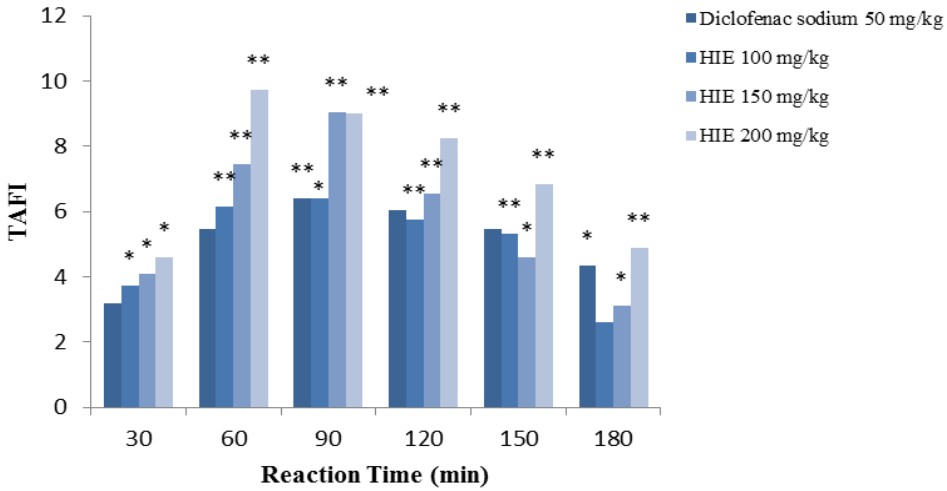

Figure 2. Tail flick antinociceptive index (TFAI) of extract of *H. integrifolia* (100, 150 and 200mg/kg) and diclofenac sodium (50mg/kg) on tail immersion pain in mice. Each point represents the mean ± SEM of 6 animals. The data was analyzed by ANOVA followed by Tukey's test. Asterisks indicated statistically significant values from control. *P < 0.05, **P < 0.01.

Table 4. Effect of extract of *H. integrifolia* on paw edema in rats

Treatment group	Dose (mg/kg)	Reaction time					
		0 h	1 h	2 h	3 h	4 h	5 h
Control	-	2.17±0.35	3.17±0.6	4.15±0.299	4.83±0.2	5.32±0.16	5.35±0.1
Diclofenac sodium	50	2.08±0.2	2.35±0.3	2.5±0.30**	2.68±0.31**	3.04±0.29**	2.7±0.2**
Extract of *H. integrifolia*	100	2.08±0.33	3.16±0.35	3.42±0.3*	3.87±0.42**	3.89±0.53**	3.96±0.45**
	150	2.08±0.32	3.03±0.47	3.36±0.30**	3.49±0.15**	3.37±0.28**	3.46±0.2**
	200	2.18±0.43	2.82±0.40	2.96±0.4*	3.15±0.40**	3.18±0.29**	3.05±.026**

Values are expressed as mean ± S.E.M (n=6). Statistical significance were calculated by ANOVA followed by Tukey's post hoc test when compare to the control group *P < 0.05, ** P< 0.0

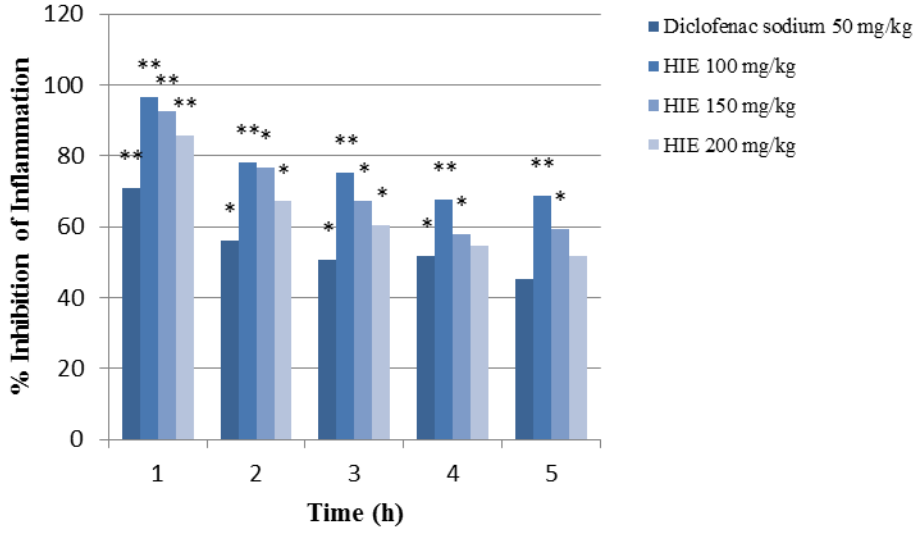

Figure 3. Percent inhibition of inflammatory of *H. integrifolia* (100, 150 and 200 mg/kg) in carrageenan induced paw edema in rats. The data was analyzed by ANOVA followed by Tukey's test. Asterisks indicated statistically significant values from control. *P < 0.05, **P < 0.01.

200 mg/kg) of extract of *H. intrigofolia* is presented in Table 4 with the average volume of the paw edema. The percent inhibition of inflammation is presented in Figure 3. The injection of the carrageenan in paw created an inflammatory edema which increased gradually. The extract of *H. intrigofolia* at the dose of 200 mg/kg exhibited an anti-inflammatory activity that became significant (P < 0.01) 2 h after the injection of carrageenan with a maximum effect of 67.17%.

DISCUSSION

Acetic acid-induced writhing is a well recommended protocol in evaluating medicinal agents for their analgesic property. The pain induction caused by liberating endogenous substances as well as some other pain mediators such as arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis (Khan et al., 2010). This pain paradigm is widely used for the assessment of peripheral analgesic activity due to its sensitivity and response to the compounds at a dose which is not effective in other methods. The local peritoneal receptor could be the cause of abdominal writhings (Khan et al., 2009). Pain sensation in acetic acid induced writhing paradigm is elicited by producing localized inflammatory response due to release of free arachidonic acid from tissue phospholipids via cyclo-oxygenase (COX), and producing prostaglandin specifically PGE2 and PGF2α, the level of lipoxygenase products may also increase in peritoneal fluids (Duarte et al., 1988). Regarding the results of extract in acetic acid-induced abdominal constriction assay, a prominent inhibition of writhing reflux was observed. These findings strongly recommend

that extract of *H. intrigofolia* has peripheral analgesic activity and their mechanisms of action may be mediated through inhibition of local peritoneal receptors which may be the involvement of cyclooxygenase inhibition potential.

Thermal nociception models such as hot plat and the tail immersion tests were used to evaluate central analgesic activity. The extract of *H. intrigofolia* showed significant ($P < 0.01$) analgesic effect in both the hot plat and tail immersion tests, implicating both spinal and supraspinal analgesic pathways. Carrageenan edema is a multimediated phenomenon that liberates diversity of mediators. It is believed to be biphasic; the first phase (1 h) involves the release of serotonin and histamine while the second phase (over 1 h) is mediated by prostaglandins, the cyclooxygenase products, and the continuity between the two phases is provided by kinins (Asongalem et al., 2004; Silva et al., 2005). Development of edema induced by carrageenan is commonly correlated with early exudative stage of inflammation (Vinegar et al., 2000). This study has shown that the methanolic extract of the fruit of *H. intrigofolia* possessed a significant anti-edematogenic effect on paw edema induced by carrageenan. Since carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents acting by the mediators of acute inflammation, the results of this study are an indication that *H. intrigofolia* can be effective in acute inflammatory disorders.

Conclusion

The methanolic extract of fruit of *H. intrigofolia* was proved as a natural safe remedy for the treatment of analgesia and inflammation. Our current findings demonstrated scientific rationale for the folk use of the plant as analgesic and anti-inflammatory. Interestingly the extract of *H. intrigofolia* exhibited both peripheral as well as central analgesic effect which might have been attributed to the presence of betulinic acid, betulin and other active principles. Nevertheless, the isolation of pure secondary metabolites from the plant will help us further in understanding the mechanism of these activities and identification of lead compounds of clinical utility.

Conflict of interest

The authors declare there are no conflict of interest concerning the publication of the manuscript.

REFERENCES

Anonymous (2001). The Ayurvedic pharmacopoeia of India .Govt of India (2201) .Misnistry of health and family welfare department of ISM and H. Part-1 pp. 39-40.

Asongalem Ea, Foyet HS, Ekoo S, Dimo T, Kamtchouing P (2004). Anti-inflammatory, lack of central analgesia and antipyretic properties of *Acanthusmontanus* (Ness) T. Anderson. J. Ethnopharmacol. 95:63-68.

Borris RP (1996). Natural products research: perspective from a major pharmaceutical company. J. Ethnopharmacol. 51(1-3):29-38.

Dharmasiri MG, Jayakody JRAC, Galhena G, Liyanage SSP, Ratnasooriya (2003). Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitex negundo*. J. Ethnopharmacol. 87:199-206.

Duarte I, Nakamura M, Ferreira S (1988). Participation of the sympathetic system in acetic acid-induced writhing in mice. Braz. J. Med. Biol. Res. 21(2):341.

Durga N, Paarakh PM (2010). Evaluation of anthelmintic activity of stem bark of *Holoptelea integrifolia* Planch. International J. Res. Ayurveda Pharm. 1:637-641.

Harleen KS, Mohanjit K, Prashant T, Manoj S, Pardeep S, Sunil P, Bimlesh K (2011). Chemistry and Medicinal properties of *Holoptelea Integrifolia*. Int. J. Drug Dev. Res. 3(1):06-11.

Hemamalini H, Soujanya LG, Vasireedy U, Pavani P (2011). CNS activity of the methanolic leaf extracts of *Sophora interrupta* Bedd, *Kigelia pinnata* Dc, *Holoptelea integrifolia* plants in experimental animal models. Int. J. Pharm. Tech. 3:3785-3791.

Hemamalini H, Sathya SB (2013). Hepatoprotective activity of *Sophara interrupta* and *Holoptelea integrifolia* against carbon tetrachloride induced hepatotoxicity in rats. Int. J. Biol. Pharm. Allied Sci. 2:80-89.

Khan MA, Khan H, Khan S, Mahmood T, Khan PM, Jabar A (2009). Anti-inflammatory, analgesic and antipyretic activities of *Physalis minima* Linn. J. Enz. Inhib. Med. Chem. 24(3):632-637.

Khan H, Saeed M, Gilani AUH, Khan MA, Dar A, Khan I (2010). The antinociceptive activity of *Polygonatum verticillatum* rhizomes in pain models. J. Ethnopharmacol. 127(2):521-527.

Koster R, Anderson M, De Bear EJ. (1959). Acetic acid for analgesic screening. Fed. Proc. 18:412-416.

Lakshmi KS, Sharma, SS, Rajesh, T, Chitra, V (2010). Antitumour activity of *Holoptelea integrifolia* on Daltonis ascetic lymphoma in Swiss albino mice. Int. J. Green Pharm. pp. 44-47.

Mahmud S, Shareef H, Ahmad M, Gouhar S and Rizwani GH (2010). Pharmacognostic studies on fresh leaves of *Holoptelea itrigofolia* (Roxb.). Pak. J. Bot. 42:3705-708.

Moody JO, Robert VA, Connolly JD, Houghton PJ (2006). Anti-inflammatory activities of the methanol extracts and an isolated furano diterpeneconstituent of *Sphenocentrum jollyanum* Pierre (Menispermaceae). J. Ethnopharmacol. 104:87-91.

Owoyele B V, Olaleye S B, Oke J M, Elegbe R A (2004). Anti-inflammatory and analgesic activities of *Nothospondias staudtii*. Niger. J. Physiol. Sci. 19(1-2):102-105.

Puri S, Bimlesh K, Jiban D, Prashant T, Manoj S, Mohanjit K, Amit M (2011). Comparative pharmacological evaluation of adaptogenic activity of *Holoptelea integrifolia* and *Withania somnifera*. International J. Drug Dev. Res. 3(1):84-98.

Rizwani GH, Mahmud S, Shareef H, Perveen R, Ahmed M (2012). Analgesic activity of various extracts of *Holoptelea integrifolia* (Roxb) Planch leaves. Pak. J. Pharm. Sci. 25:629-632.

Samsam SH, Moatar F (1991). Natural Medicines and plants, Mashal publication, Tehran, Iran.

Saraswathy AS, Devi SN, Ramasamy D (2008). Antioxidant, heavy metals and elemental analysis of *Holoptelea integrifolia* Planch. Indian J. Pharm. Sci. 70:683-576.

Sawadogo WR, Boly R, Lompo M, Some N (2006). Anti-inflammatory, analgesic and antipyretic activities of *Dicliptera verticillata*. Int. J. Pharmacol. 2:435-438.

Sharma S, Lakshmi KS (2009). Evaluation of antidiarrhoeal potentials of ethanolic extract of leaves of *Holoptelea integrifolia* in mice model. Int. J. Pharm. Tech. Res. 1:832-36.

Sharma S, Khatri, P, Pandey, A, Jakhetia, V, Chaturvedi, L, Dwivedi, N (2010). Anti-diabetic screening leaves extract of *Holoptelea integrifolia*. Int. J. Pharm. Res. Dev. 2:66-71.

Silva GN, Martins FR, Matheus ME (2005). Investigation of anti-inflammatory and antinociceptive activities of *Lantana trifolia*. J. Ethnopharmacol. 100:254-259.

Singha S, Adhikari U, Ghosh A, Chandra G (2012). Mosquito larvicidal potentiality of *Holoptelea integrifolia* leaf extract against Japanese Encephalitis Vector, *Culex vishuni*. J. Mosq. Res. 2:25-31.

Srinivas S, Ravindra K, Aradhana M, Kailash B (2008a). Ethanolic leaf extract *of Holoptelea integrifolia* Planch. decreases cisplatin-induced pica in rats. J. Pharmacogn. 7(16):293-297.

Srinivas Reddy B, Kiran Kumar Reddy R, Naidu VG, Madhusudhana K, Agwane SB, Ramakrishna S, Diwan PV (2008b). Evaluation of antimicrobial, antioxidant and wound healing potentials of *Holoptelea integrifolia*. J. Ethnopharmacol. 115:249-56.

Srinivas S, Lakshmi KS, Arjun P, Abhinav C, Sanjay D (2009). Evaluation of anti-inflammatory effect of aqueous extract of leaves of *Holoptelea integrifolia* in rats. Indian J. Pharmacol. 2(41):87-8.

Vinegar R, Schreibee W, Hugo RJ (2000). Pharmacol. Expt. Ther. 166:95-103.

Vinod NV, Shijina R, Dillep KV (2010). Inhibition of β-lactamase by 1, 4-napthalenedione from *Holoptelea integrifolia* Planch. Appl. Biochem. Biotech. 160:1752-1759.

Vinod NV, Haridas M, Sadasivan C (2010). Isolation of 1,4-naphthalenedione, an antibacterial principle from the leaves of *Holoptelea integrifolia* and its activity against β-lactam resistant *Staphylococcus aureus*. Indian J. Biochem. Biophys. 47:53-55.

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Full Length Research Paper

The role of artemether as a possible drug for treatment *Blastocystis hominis* infection: *In vivo* and *in vitro* studies

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The study is carried out to evaluate the *in vitro* and *in vivo* effect of treatment of artemether against *Blastocystis hominis* infection. In addition, transmission electron microscopic examination (TEM) and histopathological studies of *B. hominis* were carried out. The effect of artemether on *Blastocystis* viability showed reduction in viability 7 and 15% after 1 h in the group treated with 500 and700 µg/ml, respectively. There are reductions in living cell count (LCC) and living cell rate (LCR) activity of artemether on *B. hominis* isolates in 500 and 700 µg/ml, respectively in both counts. The highest concentration (1000 µg/ml) caused rapid death of the parasite. The combination of artemether + metronidazole (MTZ) showed remarkable improvement in histopathological picture. By TEM examination, *Blastocystis* cells treated with metronidazole (120 µg/ml) showed shrinking of the cytoplasm and appetites change. Blastocystis cells after exposure to 500 µg/ml artemether showed large cytoplasmic vacuoles which appeared empty with rupture of the cell wall. Finally, our studies showed that artemether can be useful as herbal candidate for *B. hominis* infection therapy.

Key words: Artemether**,** TEM examination, *Blastocystis* cells, metronidazole, histopathological picture.

INTRODUCTION

Blastocystis hominis is one of the most important protozoan parasite that affects the human intestinal tract. Various epidemiological surveys have recorded a prevalence of up to 10% of the population in developed countries and as much as 50 to 60% in developing countries. Tan (2008) and El-Shewy et al. (2002) reported the prevalence of *Blastocystis hominis* among school children in Egypt as 10%. In Dakahlia governorate (Egypt), the prevalence rate of *B. hominis* cysts reached 22% (El-Shazly et al., 2006). The parasite is commonly associated with gastro intestinal tract (GIT) symptoms such as watery and mucus diarrhea, vomiting, abdominal cramps and bloating (Kaya et al., 2007). Epidemiological and parasitological studies also suggested its role in irritable bowel syndrome (Ustun and Turgay, 2006; Stark et al., 2007; Eida and Edia, 2008).

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Blastocystis is not only found in patients with enteric symptoms, but also in healthy and asymptomatic subjects (Tan, 2004, 2008). *Blastocystis* in the human intestine shows pathogenic potential which is controversial due to foundation of organism in both symptomatic and asymptomatic individuals (Chen et al., 2003). Pathogencitiy of *B. Hominis* has been proven experimentally on guinea-pigs, which were infected either by natural way (orally) or by rectal infection of the parasite into the cecum (Boeva-Bangyozova et al., 2008). The most commonly recommended drugs for treatment of *B. hominis* and other pathogenic intestinal protozoa is metronidazole (MTZ), which was reported to cause undesirable side effects and failures in treatment (Lemee et al., 2000). The combination of trimethoprim/sulfamethoxazole with metronidazole is effective in some individuals (Moghaddam et al., 2005). The use of metronidazole as an effective drug is not recommended in intervention. The cystic form has been shown to be resistant (up to 5 mg/ml) to the cytotoxic effect of the drug. These observations may often explain the failure of treatment (Rossignol et al., 2005).

Rossignol et al. (2005) reported the effective drug against *Blastocystis* were Nitazoxanide, a 5-nitrothiazozle broad-spectrum antiparasitic agent, and cotrimoxazole. Garavelli (1991) stated that, metronidazole, iodoquinol, or co-trimoxazole are effective in *Blastocystis* infections.

Introduction of a natural antimicrobials, such as standardized oil of oregano, black walnut, artemesinin, berberine sulphate, citrus seed extract, can be of most importance (Garavelli, 1991).

Artemisia annua (sweet annie or qinghao) was naturally occurring substances having anti-protozoan activity, a plant that yields the lactone artemisinin (qinghaosu) which is the basis for a new class of anti-malarial compounds widely used in Asia and Africa (Hien and White, 1993). The anti-protozoan effects of artemisinin to its content of endoperoxides kills parasities through oxidation. The low toxicity of artemisinin and its antibiotic activity are stimulatedmacrophages, which an important componentich are important component of the immune response to protozoan infestation (Tang, 1992). Armentia et al. (1993) studied that the eradication which depend on the successful restoration of mucosal immune will function and result in continued non exposure to repeat infection.

Different concentrations (10, 100, 500 microg/ml) of *Nigella* aqueous extract and metronidazole are an active standard drug for *B. hominis* (El-Wakil, 2007). Hence, the need to develop a safe and effective alternative antiprotozoal agent is required. Artemether is active *in vitro* against the parasitic protozoa *Entamoema histolytica* and *Giardia lamblia* (Sanmi, 2012). The present study was carried out to evaluate the *in vitro* and *in vivo* effect of treatment of artemether against *B. hominis* infection. In addition, transmission electron microscopic examination (TEM) and histopathological studies of *Blastocystis hominis* were used.

MATERIALS AND METHODS

Stool samples were subjected to:

1. Direct microscopy: Stool samples were obtained from diarrheic patients attending parasitology lathe outpatient clinictient clinic of TBRI, examined by wet smear (both unstained and iodine stained) from fresh stool (Melvin and Brooke, 1974) and merthiolate iodine formaldehyde concentration technique (MIF) (Blagg et al., 1955).
2. In-Vitro cultivation of stool samples positive for *B. hominis:* Approximately 50 mg of each faecal specimen was inoculated into 2 ml of Jones' medium (0.01% yeast extract in buffer saline) (Jones, 1946), supplemented with 20% horse serum in a screw cap tube. The tubes were, then, incubated at 37°C for 48 h and a drop of the cultured solution was examined under light microscope at 10× and 40× magnifications. *B. hominis* vascular forms were found in culture specimens and were variable in size. Culture is regarded as negative when there is failure to detect *B. hominis* after 72 h (Zierdt, 1988). Stool culture was undertaken to exclude bacterial pathogens.
3. All tubes of the different group were incubated in a humidified CO2 at 37°C for ascending time intervals (30 min and up to 2 h in some assays).

(a) Parasites were counted using a Neubauer haemocytometer (Weber, England) after treatment. Total trophozoite numbers per ml in each group were calculated from the mean of at least three haemocytometer counts.
(b) Trophoziote viabilility was assessed using Eosin brilliant cresyl blue stain. The possible effect of artemether on *B. hominnis* living cell count (LCC) was assessed 30 min, 1 and 2 h and the counts were compared with those of metronidazole for the same time intervals.

Experimental animals

Twenty laboratory bred male hamsters each weighting 100 to 110 g were used. Experimental animals were kept for 3 weeks in air conditioned rooms at 21°C, receiving food containing 24% protein. The animals were supplied and housed throughout the study in Schistosome Biological Supply Center (SBSP) at Theodor Bilharz Research Institute (TBRI), an Institution responsible in animal ethics.

Infection

B. hominis cells were obtained from diarrheic patients attending parasitology laboratory, in the outpatient clinic of TBRI. Each hamster was administered *B. hominis* orally using stainless steel esophageal in a dose of 10,000 *B. hominis* cell.

Drugs

Artemether was obtained in tablet form (Kunming Pharmaceutical Cooperation, PR China) with a documented purity of 99.6%. Artemether was suspended in 7% Tween-80 and 60% (v/v) DMSO to get a final stock solution of 1 mg/ml. Serial dilutions of both drugs were performed using RPMI-1640 medium to get the desired test concentrations. Metroindazole was used as a standard drug supplied by Rhone Poulence Rorer in dose of 120 µg/ml.

Treatment

Experimental animals were divided into four groups, each

Table 1. Effect of artemether on *Blastocystis* viability using eosin-brilliant cresyl blue supravital stain.

Drug/contraction (µg/ml)	% Viable cells (1/2 h)	% Viable cells (1 h)	% Viable cells (2 h)
1% DMSO	100	100	100
Metronidazole 120	63.0	63.0	63.0
200	70.1	60	60
500	30	15	7
700	14	7	0
1000	0	0	0

Table 2. Effect of artemether (Art) on *Blastocystis* cell concentration in culture.

Drug/contraction (µg/ml)	Cells/ml±SEM (1/2 h)	Cells /ml±SEM (1 h)	Cells /ml±SEM) (2 h)
Control	100×104±4.06	100×104±4.06	100×104±4.06
1% DMSO	72.55×104±1.55	72.66×104±1.65	74.65×104±1.55
Metronidazole 120	60.72×104±1.23*	65.75×104±1.70*	52.75×104±1.90*
200	52.52×104±0.86*$^{+}$	35.35×104±1.41*$^{+}$	32.35×104±1.27*$^{+}$
500	40.5×104±2.02*	30.25×104±1.25*$^{+}$	21.25×104±1.20*$^{+}$
700	25.5×104±0.44*$^{+}$	13.25×104±2.35*$^{+}$	0
1000	0	0	0

*Significant difference between treated group and control groups ($P < 0.05$). $^{+}$Significant difference between treated group(Contraction 200) and control groups ($P < 0.05$)

comprising 3 hamsters. Animals were administered artemether orally using stainless steel oesophogeal for 3 consecutive days in a dose of 400 mg/kg/day. Animals received metronioazole (supplied by Rhone Poulence Rorer) in a dose of 400 µg/kg body weight. Animals of all four groups were sacrificed 3 week post infection.

Experimental design

Group 1: infected untreated group (control) group.
Group II: hamsters receiving metronioazole (supplied by Rhone Poulence Rorer) in a dose of 400 µg/kg body weight. Group III: hamsters were given Artemether in a dose of 400 mg/kg/day for 3 consecutive days.
Group VI: Infected-treated with a combination of 1/3 dose of metronioazole that is, 100 µg/kg body weight) plus artemether a dose of 300 mg/kg/day for 3 consecutive days.

Parasiological analysis

Three weeks following infection, treatment was given. Two weeks later stool analysis was performed by the merthiolate iodine formaldhyde concentration (MIFC) technique (Blagg et al., 1955) *Blastocystis* cell which is vacuolated from cells/ml were counted.

Transmission electron microscopic examination (TEM)

From culture sediment after receiving different drugs was done as a trial to discuss the mode of action of these drugs. Samples from different groups were fixed in 4% glutaraldehyde in 0.1 sodium cacodylate buffer to be studied by TEM according to Grimaud et al. (1980). TEM examination was performed in the electron microscopy lab at TBRI.

Histopathological studies

After scarification of hamsters, the small bowel was removed. A 1 cm segment was excised 5, 15 and 25 cm from the gastroduodenal junction. The excised segment was opened longitudinally and finally embedded in paraffin wax. Transverse sections of paraffin blocks were cut by microtome and mounted on glass slides. Thickness 5 µm deparaffinization sections were performed by dipping slides in 100% xylene and descending grades (100, 95, 80 and 70%) of ethanol for rehydration. Finally, sections were stained with Enrlich's hematoxylin and counter stained with eosin. Five slides/animal and 3 sections/slide in each group were prepared. Histological section (3 to 5 µm) were stained with hematoxylin-eosin lymph nodes (Peyer's patches) and spleens were also examined (Bancroft and Stevens, 1975).

Statistical analysis

Statistical analysis of the results was carried out using one-way analysis of variance (ANOVA) according to Campbell (1989). Comparison between two groups was done by the Student' test. The data were considered significant if $P < 0.05$.

RESULTS

Table 1 shows the effect of artemether on *Blastocystis* viability using eosin-brilliant cresyl blue supravital stain. It shows reduction in viability 7 and 15% after 1 h in the group treated with 500 and700 µg/ml, respectively. Table 2 shows living cell count (LCC) and living cell rat (LCR) activity of artemether showing reduction in LCC of *B.*

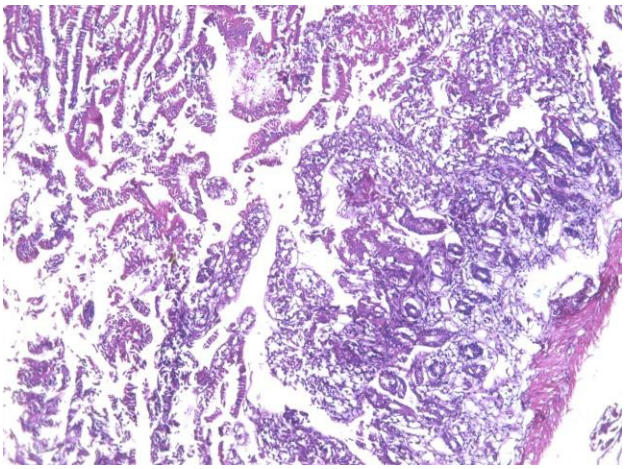

Figure 1. Intestine of control infected group, lined by columnar mucin secreting cells with partial loss of villouus pattern showing erosions and ulcers . Lamina and core of villi are infiltrated by large number of chronic inflammatory cells (H&E ×100).

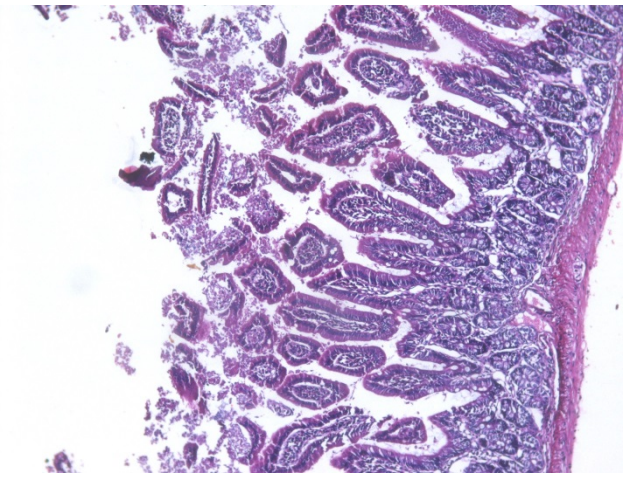

Figure 3. Intestine of treated group with Artmether lined by columnar mucin secreting cells with presentation of normal villous pattern showing focal superficial erosions (short arrow). The lamina and core of villi are infiltrated with moderate number of chronic inflammatory cells (H&E ×200).

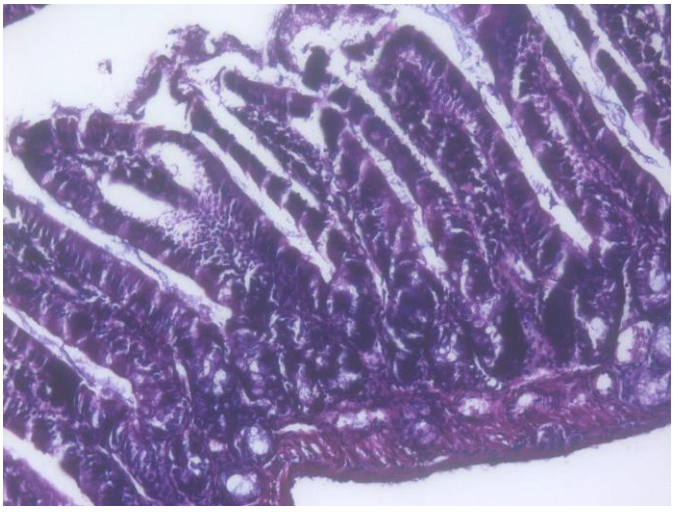

Figure 2. Intestine of treated group with Metronidazole lined by columnar mucin secreting cells with presentation of villous pattern showing focal minute ulceration of the lining epithelium. lamina and core of villi connective tissue is infiltrated by mild number of chronic inflammatory cells (H&E ×100).

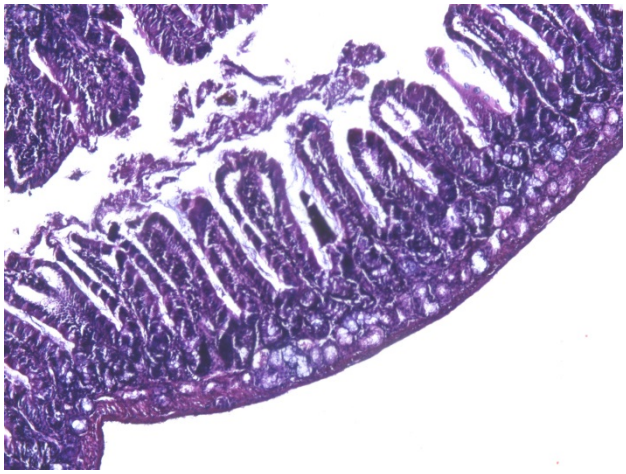

Figure 4. Artmether + Metronidazole: Intestinal mucosa showed, normal architecture . The villa are lined by mucin secreting cells with no erosions & ulcers. The lamina and villi cores are infiltrated by mild number of chronic inflammatory cells (H&E, ×200).

hominis isolates in 500 and 700 µg/ml, respectively. The highest concentration (1000 µg/ml) caused rapid death of the parasite. Metronidazole produced less reduction in mean number of count 52.75 cells/ml of *Blastocystis* cells in culture which was after 2 h.

Histopathological study

Figure 1 shows intestine of control infected group, lined by columnar mucin secreting cells with partial loss of villouus pattern showing erosions and ulcers. Lamina and core of villi are infiltrated by a large number of chronic inflammatory cells (H&E ×100). Figure 2 shows intestine of the treated group with metronidazole lined by columnar mucin secreting cells with presentation of villous pattern showing focal minute ulceration of the lining epithelium. Lamina and core of villi connective tissue are infiltrated by mild number of chronic inflammatory cells, these lead to partial hilling of intestine (H&E ×100). Figure 3 shows intestine of the treated group with artmether lined by columnar mucin secreting cells with presentation of normal villous pattern showing focal superficial erosions. The lamina and core of villi are infiltrated with moderate number of chronic inflammatory cells, these lead to partial hilling of intestine (H&E ×200). Figure 4 shows

Figure 5. (a) Control Ultrastructure of *B. hominis*. TEM showing *B. hominis* cell displaying normal morphology and DNA chromatin seen as a crescentic mass (×14000) Control. (b) Ultrastructure of *B. hominis*. TEM showing *B. hominis* cell displaying normal morphology and DNA chromatin seen as a crescentic mass (×14000) Control.

artmether + metronidazole. Intestinal mucosa showed normal architecture. The villa is lined by mucin secreting cells with no erosions and ulcers. The lamina and villi cores are infiltrated by mild number of chronic inflammatory cells (H&E ×200). It shows remarkable improvement of histopathological study.

Transmission electron microscopic (TEM) studies

Figure 5 *shows* TEM *B. hominis* cell displaying normal morphology and DNA chromatin seen as a crescentic mass (×14000) control. Figure 6 shows TEM *Blastocystis* cell treated with metronidazole (120 µg/ml) showing

concentration (200, 500, 700 and 1000 µg/ml) at different time intervals 1/2, 1 and 2 h) were studied. In this study, artemther at 500 and 700 µg/ml induced highly significant reduction in the number of concentrations of *Blastocystis* cells after 2 h incubation ($p < 0.01$). Higher concentration (1000 µg/ml) caused rapid death of the parasite and no viable cells were detected after 30 min. Our results agreed with El-Wakil (2007) who proved that *N. sativa* aqueous extract could be useful in the treatment of *B. hominis.* This study is an arrangement with Hassan et al. (2010) who stated that, dodecanoic acid (monolaurin) induced highly significant reduction of viability of *B. hominis* cells, 30 and 20% in culture after 2 h incubation at 500 and 700 µg/ml concentration, respectively at ($p < 0.01$). The highest concentration (1000 µg/ml) caused rapid death of the parasite and no viable cells were detected after 30 min incubation. Shrinking of *B. hominis* cells were observed using direct microscopy ×100 following incubation with monolaurin. TEM studies of *B. hominis* cells displayed key morphological features of programmed cell death, viz., nuclear condensation and DNA in nucleus showed reduced cytoplasmic volume with maintenance of plasma membrane integrity.

El-Gayar and Soliman (2011) studied the effect of Quillaja saponaria (QS) against cultures of *B. hominis* and demonstrated its implementation on ultrastructure changes. They found that the QS (1000 µg/ml) produced a high significant reduction in both living cell counts (LCC) and living cell rate (LCR) on day 1 compared to the control. Zierdt (1991) stated that an active drug (metronidazole) concentration should inhibit the growth of *B. hominis.* Sawangjaroena and Sawangjaroen (2005) recorded that at a concentration of 2000 µg/ml the three extracts *Acacia catechu* (Fabaceae) resin, *Amaranthus spinosus* (Amaranthaceaae) and *Brucea javanica* (simaroubaceae) killed 82, 75 and 67% of *B. homninis* parasites tested, respectively and was used as ant diarrheic agents against *B. homninis*.

Mahmod et al. (2006) studied the effect of the antimalarial drug artesunate, in intestinal giardiasis. He found that, the double dose of artesunate (100 mg/kg) gives the lowest mean number of Giardia (0.7 ± 1.1) and completed regeneration of endothelial cells in intestinal villi. This could be of utmost help in endemic areas like Egypt, where *Giardia lamblia* intestinalis is a common parasitic infection and resistance to the commonly used antigiardial drugs commonly known as supervenes. In our histopathological study, best results were obtained in the group receiving Artemether + MTZ. In Figure 4, intestinal mucosa showed normal architecture. The villa is lived by rows of early epithelial cell with no erosions and alces. The lamina and villi cones are unfiltered by needle under number of chronic inflammatory cells . This means remarkable improvement in histopathological picture. El-Gayar and Soliman (2011) found a remarkable improvement in the intestinal histopathological findings, the mucosa was infiltrated with few acute inflammatory cells, the lining columnar epithelial cells were intact and the submucosa was free from inflammation, in infected rats receiving 500 µg/ml. Quillaja saponaria (QS) was observed in comparison with those receiving MTZ.

Zhang et al. (2006) showed that when metronidazole was used (120 µg/ml), TEM observation indicated a reduction of microvilli on the surface of absorptive cells. Mitochondrial edema, rough endoplasmic reticulum dilatation and degranulation were found on absorptive cells and goblet cells. Lymphocyte infiltration and eosinophilia were found in intercellular stroma. These results agree with our results which indicated that *Blastocystis* cell treated with metronidazole (120 µg/ml) showed shrinking of the cytoplasm and apoptotic change (Figure 6). In this study, blastocystis cell after exposure to 500 µg/ml artemether showed large cytoplasmic vacuoles that appeared empty and a ruptured cell wall (Figure 7).

This agreed with El-Gayar and Soliman (2011), in which their study on QS proved that *B. hominis* programmed cell death with appotic-like features and concluded that QS is a promising new herbal therapeutic agent against *B. hominis* infection.

In this study, histopathological studies showed significant improvement in the experimental hamster receiving artemether comparable to MTZ. The effect of artemether on *Blastocystis* viability showed reduction in viability 7 and 15% after 1 h in the group treated with 500 and700 µg/ml, respectively. There is reduction in living cell count (LCC) and living cell rate (LCR) activity of artemether on *B. hominis* isolates in 500 and 700 µg/ml, respectively. The highest concentration (1000 µg/ml) caused rapid death of the parasite. The combination of artemether + MTZ showed remarkable improvement in histopathological picture. By TEM examination, *Blastocystis* cell treated with metronidazole (120 µg/ml) showed shrinking of the cytoplasm and apoptotic change.

Blastocystis cell after exposure to 500 µg/ml artemether showed large cytoplasmic vacuoles which appeared empty in ruptured cell wall.

Conclusion

Artemether can be useful as herbal candidate therapy for *B. hominis* infection therapy.

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Conflict of interest

Authors have none to declare.

REFERENCES

Armentia A, Mendez J, Gomez A, Sanchis E, Fernandez A, De la Fuente R, Sanchez P (1993). Urticaria by <I>*Blastocystis hominis*</I>. Succe ssful treatment with paromomycin. Allergologia et Immunopathologia 21:149-151.

Bancroft JD, Stevens A (1975). Histopathological Stains and their Diagnostic Uses. Churchill Livingstone, Edunberg, London and New York pp. 25-46.

Basuldo JA, Cordoba MA, de Luca MM, Ciarmela ML, Pezzani BC, Grenovero MS, Minvielle MC (2007). Intestinal parasitoses and environmental factors in a rural population of Argentina, 2002-2003. Rev. Inst. Med. Trop Sao Paulo 49:251-255.

Blagg W, Schloegel EL, Mansour NS, Khalaf GJ (1955). A new concentration technic for the demonstration of protozoa and Helminth eggs in feces. Am. J. Trop. Med. Hyg. 4:23-28.

Boeva-Bangyozova V, Panayotova M, Chakarova B (2008). Blastocystosis: Pathogenesis, clinical course, diagnostics and treatment. Trakia J. Sci. 6:94-97.

Calzada F, Yepez-Mulia L, Tapia-Contreras A (2007). Effect of Mexican medicinal plant used to treat trichomoniasis on Trichomonas vaginalis trophozoites. J. Ethnopharmacol. 113(2):248-51.

Campbell RC (1989). Statistics for Biologists. 3rd Edn., Cambridge University Press,. Cambridge, UK p 446.

Chen TL, Chan CC, Chen HP, Fung CP, Lin CP, Chan WL, Liu CY (2003). Clinical Characteristics and endoscopic findings associated with <I>Blastocystis hominis</I> in healthy adults. Am. J. Trop. Med. Hyg. 69:213-216.

Dinleyici EC, Eren M, Dogan N, Reyhanioglu S, Yargic ZA, Vandenplas Y (2011). Clinical efficacy of <I>*Saccharomyces boulardii*</I> or metronidazole in symptomatic children with <I>*Blastocystis hominis*</I> infection. Parasitol. Res. 108:541-545.

Eida AM, Eida MM (2008). Identification of <I>*Blastocystis hominis*</I> in patients with irritable bowel syndrome using microscopy and culture compared to PCR. Parasitol. United J. 1:87-92.

El-Shewy KA, El-Hamshary EM, Abaza SM, Eida AM (2002). Prevalence and clinical significance of *Blastocystis hominis* among school children in Ismailia city. Egypt. J. Med. Sci. 23:31-40.

El-Wakil SS (2007). Evaluation of the in vitro effect of Nigella sativa aqueous extract on Blastocystis hominis isolates. J. Egypt Soc. Parasitol. 37:801-813.

El-Gayar EK, Soliman RH (2011). *In vitro* and *in vivo* effects of Quillaja on *Blastocystis hominis* isolates. PUJ 4:101-108.

El-Shazly AM, Awad SE, Sultan DM, Sadek GS, Khalil HH, Morsy TA (2006). Intestinal parasites in <I>*Dakahlia governorate*</I>, with different techniques in diagnosing protozoa. J. Egypt. Soc. Parasitol. 36:1023-1034.

Garavelli PL (1991). The therapy of blastocystosis. J. Chemother. 3:245-246.

Grimaud JA, Druget M, Peyrol S, Chevalier D (1980). Collageimmuno-typing in human liver: Light and electron microscope study. J. Histochem. Cytol. 28:1145-1151.

Hassan SI, Moussa HME, Fahmy ZH, Mohamed AM (2010). In Vitro the effect of medium-chain saturated fatty acid (dodecanoic acid) on *Blastocytsis* spp. Int. J. Infect. Dis. 14:584.

Hien TT, White NJ (1993). Qinghaosu. Lancet 341:603-608.

Hussien A, Virmami OP, Popli SP (2008). Dictionary of Indian Medicinal Plants. CIAP, Lucknow, India p 2384.

Jones WR (1946). The experimental infection of rats with Entamoeba histolytica; with a method for evaluating the anti-amoebic properties of new compounds.. Ann. Trop. Med. Parasitol. 40:130-140.

Kaya S, Cetin E, Aridogan BC, Arikan S, Demirci M (2007). Pathogenicity of <I>*Blastocystis hominis*</I>, a clinical reevaluation. Turkiye Parazitol. Derg 31:184-187.

Lemee V, Zaharia I, Nevez G, Rabodonirina M, Brasseur P, Ballet JJ, Favennec L (2000). Metronidazole and albendazole susceptibility of 11 clinical isolates of <I>*Giardia duodenalis*</I> from France. J. Antimicrob. Chemother. 46:819-821.

Mahmod SS, Guirgus N, Helmy A, Sabry H (2006). Effect of a novel antimalarial drug on different pathological and parasitological parameters in experimental intestinal giardiasis. New Egypt. J. Med. 35:38-43.

Melvin DM, Brooke MM (1974). Laboratory Procedures for the Diagnosis of Intestinal Parasites. 2nd Edn., U.S. Department of Health, Education and Welfare, Washington, DC., USA p 199.

Moghaddam DD, Ghadirian E, Azami M (2005). <I>*Blastocystis hominis*</I> and the evaluation of efficacy of metronidazole and trimethoprim/sulfamethoxazole. Parasitol. Res. 96:273-275.

Nigro LL, Larocca L, Massarelli I, Patamia I, Minniti S, Palermo F, Cacopardo B (2003). A placebo-controlled treatment trial of *Blastocystis hominis* infection with metronidazole. J. Travel Med. 10:128-130.

Ozcakir OS, Gureser S, Erguven YA, Yilniaz R, Topaloglu R, Hascelik G (2007). Characteristics of *Blastocystis hominis* infection in a Turkish university hospital. Turkiye Parazitol. Derg. 31:277-282.

Rayan HZ, Ismail OA, El Gayar EK (2007). Prevalence and clinical features of *Dientamoeba fragilis* infections in patients suspected to have intestinal parasitic infection. J. Egypt. Soc. Parasitol. 37:599-608.

Rohner K, Demuth D (1994). Adverse effects of veterinary drugs. Schweiz Arch. Tierheilkd. 136:309-312.

Rossignol JF, Kabil SM, Said M, Samir H, Younis AM (2005). Effect of nitazoxanide in persistent diarrhea and enteritis associated with *Blastocystis hominis*. Clin. Gastroenterol. Hepatol. 3:987-991.

Sanmi AA (2012). Oxidative stress in biochemical, seminological and histological alterations due to acute administration of intramuscular artemether in mice. Researcher 4:9-17.

Sawangjaroena N, Sawangjaroen B (2005). The effect of extracts from anti-diarrheic Thai Medicinal plants on the *in vitro* growth of the intestinal protozoa parasite: Blastocystis hominis. J. Ethnopharmacol. 98:67-72.

Stark D, van Hal S, Marriott D, Ellis J, Harkness J (2007). Irritable bowel syndrome: A review on the role of intestinal protozoa and the importance of their detection and diagnosis. Int. Parasitol. 37:11-20.

Stensvold CR, Smith HV, Nagel R, Olsen KE, Traub RJ (2010). Eradication of *Blastocystis* carriage with antimicrobials: Reality or delusion? J. Clin. Gastroenterol. 44:85-90.

Tan KSW (2004). <I>*Blastocystis*</I> in humans and animals: New insights using modern methodologies. Vet. Parasitol. 126:121-144.

Tan KSW (2008). New Insights on classification, identification and clinical relevance of *Blastocystis* spp. Clin. Microbiol. Rev. 21:639-665.

Tang W (1992). Chinese Drugs of Plant Origin. Springer Verlag. Berlin. pp. 160-174.

Tasova Y, Sahin B, Koltas S, Paydas S (2000). Clinical significance and frequency of *Blastocystis hominis* in Turkish patients with hematological malignancy. Acta Medica Okayama 54:133-136.

Ustun S, Turgay N (2006). *Blastocystis hominis* and bowel diseases. Turkiye Parazitol. Derg. 30:72-76.

Valsecchi R, Leghissa P, Greco V (2004). Cutaneous lesions in <I>*Blastocystis hominis*</I> infection. Acta Derm. Venereol. 84:322-323.

Zhang HW, Li W, Yan QY, He LJ, Su YP (2006). Impact of *Blastocystis hominis* infection on ultrastructure of intestinal mucosa in mice. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi, 24:187-191.

Zierdt CH (1988). *Blastocystis hominis*, a long-misunderstood intestinal parasite. Parasitol. Today 4:15-17.

Zierdt CH (1991). *Blastocystis hominis* past and future. Clin. Microbiol. Rev. 4:61-79.

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Review

Toxicity of food colours and additives: A review

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Majority of consumer goods are required to be presented with good aesthetics in order to improve acceptability in terms of colours and in some instances taste. When related to food, beverages and drug products, additives are usually added to mask un-inviting colours, obscure offensive odours and increase taste. Food additives therefore include colourants, sweeteners, preservatives and anti-caking agents. Admissible daily intake limits are often recommended for these additives. Being food products, the amount consumed over time may be subject to individual preferences and thus negating the desire to regulate and control the amount consumed cumulatively. There have been several concerns about the safety of food additives and several batteries of tests, and reports are available in literature. This review attempted to give an update on reports that have surfaced in literature over recent past on the use and safety of food colours and other additives. Some safety concerns have been related to three determinations; cytotoxicity, genotoxicity and induction or potential of inducing mutagenicity. In order to accomplish these targeted evaluations, several tests have been prescribed by International conference on harmonization (ICH), organization for economic co-operation and development (OECD) and European food safety authority (EFSA). It is observed that no single test can give a full proof of safety of these food colours and additives, hence minimal tests are recommended to be carried out in order to guarantee safety of these products. Survey of literature, revealed that once some approved additives or colours become a subject of safety concerns, comprehensive evaluations are carried out by researchers and these have often led to the de-classification of some hitherto reported agents as being non-genotoxic or non-carcinogenic. The declassifications of some food colors and additives as human carcinogens are regularly done following the comprehensive evaluation of results of mutagenicity and genotoxicity tests *in vitro* and some *in vivo* tests in mammalian tissues and whole animals. However, such declassifications are often done with caution and the implication is that regular and more comprehensive tests must be carried out. In addition, the requirements of testing for chronic exposures to this and other agents must be emphasized to prevent occurrence of subtle yet terrible side effects resulting from consuming sub-toxic doses of the additives over time.

Key words: Food colour, food additive, cytotoxicity, genotoxicity, mutagenicity, safety concerns.

INTRODUCTION

Food additives are substances that are deliberately added to food substances to impart desired characteristics. They are used for various purposes including preservation, aesthetics, taste masking and sweetening

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(Yu et al., 2002; Güngörmüş and Kılıç, 2012). However, several epidemiological studies have established an association between environmental exposure to various anthropogenic substances and the development of cancer especially, bladder cancer in later life (Golka et al., 2004).

The ability of drugs and additives to cause genotoxic and/or carcinogenic effects was first reported in the 1970s amongst textile and hair dye applicators (Fraumeni Jr and Miller, 1972; Brambilla and Martelli, 2009). The benzidine-based dyes in use then were reported to cause an increased risk of up to 6.4 times than the expected rate of developing bladder cancer later in life (Golka et al., 2004). Following the sulphanilamide elixir and thalidomide crisis in the 1930s and 60s respectively, researchers like Rall (1975) regarded carcinogenicity of drugs as the next potential drug regulatory crisis and strongly advocated for their continuous evaluation using lifetime rodent carcinogenicity assay. The recommended standard battery of tests for the genotoxicity assessment of new chemical entities includes:

(1) Salmonella gene mutation test.
(2) *In vitro* gene mutation test using human lymphocytes.
(3) A test for chromosomal aberration and DNA damage *in vivo* using rodent bone marrow (FDA, 1993).

The objectives of genotoxicity tests are to detect mutagens and carcinogens as well as study the mechanisms of chemical mutagenesis and carcinogenesis; and assess the mutagenic and carcinogenic hazards of chemicals to humans Until recently, the end points of the most frequently used methods usually employ the detection of either gene mutation in bacteria or specialized mammalian cells, or the *in vivo* and *in vitro* assessment of chromosomal aberrations in *in vitro* and *in vivo* models or systems (Sasaki et al., 2000).

The current international conference on HARMONIZATION (ICH) guidelines on genotoxicity of small molecules allow for a standard battery of tests at both *in vitro* and *in vivo* interphases (ICH). The tests are designed and aimed at detecting compounds that induce genetic damage by various mechanisms. “These tests enable hazard identification with respect to damage to DNA and its fixation. Fixation of damage to DNA in the form of gene mutations, larger scale chromosomal damage or recombination is generally considered to be essential for heritable effects and in the multi-step process of malignancy, a complex process in which genetic changes may play only a part” (ICH S2(R2)).

This review attempts to chronicle the various food additives that are currently accepted as ‘safe’ by either the food and drug administration (FDA or USFDA) or the organization for economic co-operation and development (OECD). The review also covers some independent studies that have been carried out on the food additives and thereafter presents an overview of current status on the genotoxicity or otherwise of these approved food additives.

Approved food colours and additives

The US FDA and the European Union as well Canadian agency have all equally published a list of approved colourants and additives. The documents from these agencies are comprehensive and there are guidelines to the use of agents meant for use in animal and man. In the FDA Federal register, agents approved for animal use are clearly identified and those allowed for human consumption are also prescribed. The FDA guide stipulates that “color additives listed in 21 CFR Parts 74 and 82 must be analyzed and batch certified by FDA before they can be used in any FDA-regulated product marketed in the U.S. This requirement applies to products imported into this country as well as those manufactured domestically” (FDA, accessed 20 May, 2015). In addition, the document stipulates that “manufacturers of certified color additives must include on the label the name of the certified color additive, a statement indicating general use limitations, any quantitative limitations in products, and the certification lot number assigned to the batch”.

The EU document last updated in 2014 also stipulates that “Most additives are only permitted to be used in certain foods and are subject to specific quantitative limits, so it is important to note this list should be used in conjunction with the appropriate European Union legislation” (EU, accessed 20 May, 2015). However, a proviso clearly states that the document is not for legal guidance. Each approved food colour and additives are assigned specific “E” numbers. The following sections of this review provide a comprehensive review and assessment of literature available on approved colourants and other additives. The data available from researchers on the three indices of toxicity (cytotoxicity, genotoxicity and mutagenicity) are reviewed. The chemical structures of the food colours whose toxicity profiles are reviewed are presented in Figure 1.

SUMMARY OF LITERATURE ON THE GENOTOXICITY ASSESSMENT OF SOME FOOD ADDITIVES

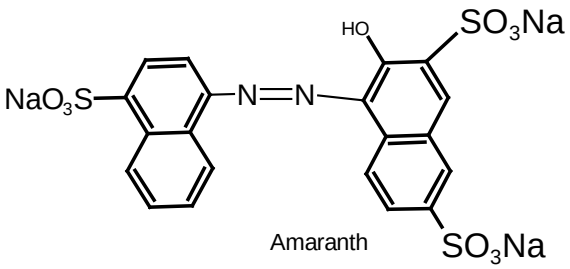
Amaranth

Food Red No.2, CAS No. 915-67-3.
Chemical name: Trisodium 2-hydroxy-1-(4-sulphonato-1-naphthylazo)naphthalene-3,6-disulphonate.

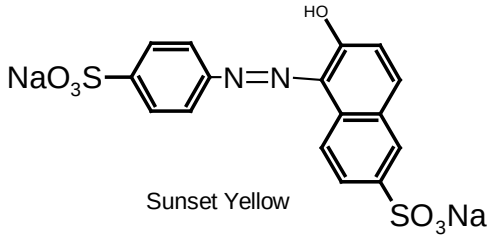
Cytogenetic evaluation

Amaranth in an *in vitro* assay increased the frequency of

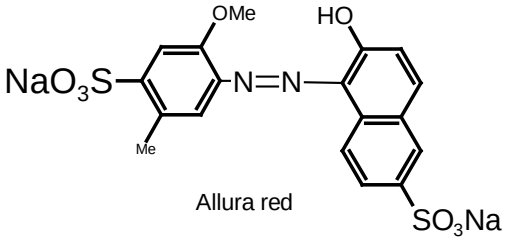
(a) **Azo dyes**



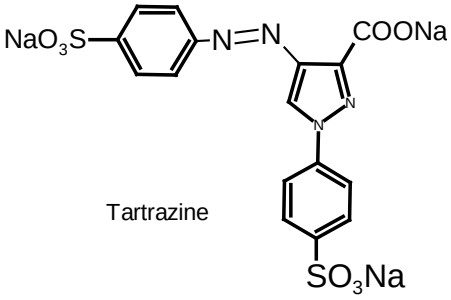
Amaranth



Sunset Yellow

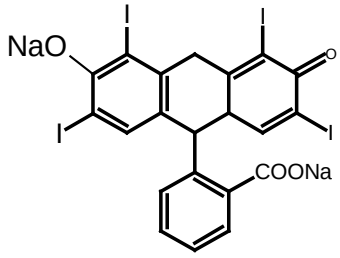


Allura red



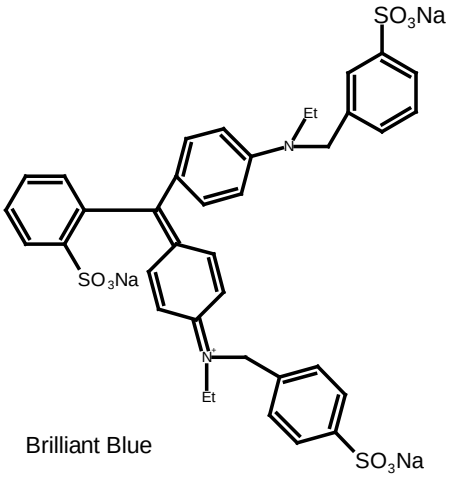
Tartrazine

(b) **Xanthene dyes**



Erythrosine

(c) **Triphenymethane dyes**



Brilliant Blue

Figure 1. Some representative food colours.

sister chromatid exchange in treated cells compared to control. It also demonstrated cell cytostatic potential (Mpountoukas et al., 2010). There is an abundance of old literature on amaranth because of its long history of use in the food and drug Industry (Combes and Haveland-Smith, 1982; Leconinte and Lesca, 1978). There are many reports documenting the lack of genotoxicity of amaranth on bacteria (Brown et al., 1978; Haveland-Smith and Combes, 1980; Chung et al., 1981; Das and Mukherjee, 2004).

However, there is still a large amount of uncertainty and discrepancies on the reported *in vivo* follow up tests. Sasaki et al. (2002) reported the genotoxicity of amaranth using comet assay on eight mouse organs. Following a

24 h single dose treatment, amaranth at doses lower than the admissible daily intake (2000 mg/kg body weight, (b.w.)) showed genotoxicity damage in three different organs. These included tests aimed to expose specific colon DNA damage at doses as low as 10 mg/kg b.w. after a 3 h sampling time (Yu et al., 2002). Results from a similar study are corroborative but seem to suggest inter-species differences in toxicity towards mice and rats (Shimadaet et al., 2010).

On the other hand, the dye as assessed by the micronucleus gut assay of mice following double dosing at 24 h interval was reported to be non-mutagenic and did not show any significant difference in the frequency of micronucleated cells relative to control. The authors went on to suggest that the toxicity observed in Sasaki et al. (2000) report might be due to acute cell toxicity and death following treatment with the dye (Poul et al., 2009). Similar results were obtained in another study that involved the micronucleus assay of the stomach, colon and bone marrow of rats (Okada et al., 2013). The inability of the colourant to induce chromosomal aberrations in *in vivo* bone marrow assay has also been reported (Das and Mukherjee, 2004).

There is insufficient evidence to confirm carcinogenicity of amaranth in humans and it is presently unclassified as a human carcinogen. Such declassifications for food colours and additives are, however, often done with caution and the implication is that regular and more comprehensive tests must be carried out with very large sample size in order to be able to draw unequivocal conclusions of toxicity or otherwise. In addition, the requirements of testing for chronic exposures to these agents must be emphasized to prevent occurrence of subtle yet terrible side effects resulting from consuming chronic and sub-toxic doses of the additives over time.

Tartrazine

FD&C Yellow No. 5, Food Yellow No.4, E 102, CAS 1934-21-0.
Chemical name: 3-carboxy-5-hydroxy-1-(4'-sulphophenyl)-4-(4'-sulphophenylazo) pyrazole trisodium salt.

Cytogenetic evaluation

In a recent study, the interaction of tartrazine and endogenous material like bovine haemoglobin was described as spontaneously involving Van der Waal's forces and hydrogen bonds between the oxygen atoms at position 31 and 15 in the dye (Li et al., 2014). In a similar study, tartrazine showed extensive DNA binding, cytostatic potential and reduced mitotic index (Mpountoukas et al., 2010).

Gene mutation assays

Several reports have indicated the non-mutagenicity of tartrazine using *Salmonella typhimurium* and *Escherichia coli* (Chung et al., 1981; Das and Mukherjee, 2004; Elhkim et al., 2007; EFSA, 2009). The addition of rat liver microsome preparation S9 have also not shown the colourant as genotoxic (EFSA, 2009). In a *Salmonella* mutagenicity assay without metabolic activation, Das and Mukherjee (2004) reported the inability of the dye (at 10 to 1000 µg/plate concentration) to induce point mutation in Tester strain T97a and base pair mutation in T100. However, there was a significant positive result with the strain T98 but this was not dose-related.

In vivo assays

Sasaki et al. (2002) reported the results of comet assay on eight mouse organs following oral administration of tartrazine up to doses of 2000 mg/kg b.w. After only 3 h post-administration, DNA damage was reported at dose levels of 10 mg/kg b.w in the colon and the glandular stomach at doses higher than 10 mg/kg b.w. (Sasaki et al., 2002). Poul et al. (2009) demonstrated the non-mutagenicity of tartrazine when administered as oral gavage up to doses of 2000 mg/kg b.w. The dye at any of the tested doses did not produce an increase in the number of micronucleated colonic cells when compared with water and olive oil control groups. The food dye however produced an increase in mitotic index at all tested doses although this effect was not dose related. The authors thereafter concluded that the extensive DNA damage observed by Sasaki et al. (2002) at the same dose level could be due to the acute cytotoxicity of the dye. It could also be due to insufficient DNA repair at the 3 h sampling time (Poul et al., 2009).

Chronic toxicity

Long term carcinogenicity tests

In order to assess the effects of tartrazine on chronic administration, some reports are currently available in the literature. A 90-day oral sub-chronic toxicity involving daily dosing of Wistar rats has been reported. The rats were divided into groups of six and fed a diet containing 5, 7.5 and 10 mg/kg b.w. The haematological, clinical chemistry and histopathology parameters of the treated animals were then compared with a control group that received only distilled water. The results showed significant dose-related increase in blood chemistry parameters like glucose, creatinine, blood urea nitrogen, total cholesterol, total serum protein, triglycerides and aspartate aminotransferase when compared with the control group (Himri et al., 2011). A lack of carcinogenicity

in F334 rats have been reported when tartrazine was administered in doses of 0, 1 and 2% in the drinking water of groups of 50 male and female rats for 2 years (Maekawa et al., 1987). No toxic lesions were reported in the treated groups at any of the dye doses. All of the tumours observed, both in the treated and control groups, were characteristically spontaneous in this strain of rats and the authors did not find any difference in the organ distribution and the histology of the affected organs when they compared the treated groups with the control. Similarly, when compared with the incidences of spontaneous mesothelioma (amongst males) and endometrial stromal polyps (in females) derived from historical controls; the authors concluded that the increase in the incidences of these tumours that was observed in the 1% treated group is not related to the dye administration.

Reproductive and developmental toxicology

The teratogenic potential of dose levels of 0, 60, 100, 200, 400 and 600 mg/kg b.w of tartrazine when administered to pregnant Osborne-Mendel within the first 19 days of gestation has been reported. Tartrazine when presented either as oral gavages or in drinking water did not produce any significant toxic effect in the treatment groups when compared with the control groups. The primary outcomes used to assess teratogenicity included maternal clinical findings, rate/success of implantation, foetal size and development, foetal bone and visceral organ development (Collins et al., 1990, 1992).

Mehedi et al. (2009) however, reported the toxicity of tartrazine on the reproductive organs of male Swiss albino mice which were administered doses of 0, 0.1, 1.0 and 2.5% in drinking water for 13 weeks. The results showed that although there was no significant difference in the weights of the seminal vesicle and epididymis of the treated animals, a reduced sperm count and increased incidence of sperm abnormalities were observed in the treatment group that received 2.5% dose. They also reported a reduction in reproductive performance as estimated by mating index in the groups that received 1 and 2.5% doses.

Hyperactivity, anxiety and depression effects

Kamel and El-lethey (2011) reported that tartrazine caused an increased activity, anxiety and anti-social behaviour among male Wistar rats. The rats were put in three groups of 15 and given tartrazine doses of 0, 1 and 2.5% in their drinking water. The treated animals showed an increase in anxiety as measured by the different animal models of open field, elevated plus-maze and the dark-light transition tests. Hyperactivity, as measured with the open maze model, depression and a decreased frequency of social contacts in the treated groups was also reported.

Allura Red

FD & C Red 40, CAS No. 25956-17-6.
Chemical Name: disodium 2-hydroxy-1-(2-methoxy-5-methyl-4-sulphonatophenylazo) naphthalene-6-sulphonate.

Allura red was first introduced in the USA as a food colourant in 1971. It is non-genotoxic in many gene mutation tests involving prokaryotic and eukaryotic cells with or without activation (Chung et al., 1981; Combes and Haveland-Smith, 1982). However, allura red was reported to show direct genotoxic effect when different concentrations of the dye ranging from 9.76 to 5000 µg/mL was incubated with a culture of *Saccharomyces cerevisiae* at 37°C. Comet assay revealed dose-related DNA damage starting at concentration of 1250 µg/ml, though no positive correlation could be established with exposure time (Jabeen et al., 2013).

In vivo assays

The non-teratogenicity of allura red has been reported after groups of 11 day old pregnant rats were fed up to 2000 mg/kg b.w. of single oral doses of allura red (Tsuda et al., 2001). Comet assay was used to assess DNA damage in the embryo at 3, 6 and 24 h sampling times. When assessed by comet assay, colon-specific DNA damage was reported in mice at doses of 10 mg/kg b.w. three hour post-administration but no damage was observed in rats at any of the tested dye doses or exposure times (Shimada et al., 2010).

The results of the latter study are partly responsible for the somewhat surprising European Food Safety Authority (EFSA) classification of allura red as prohibited in animal feeds though it is still regarded as safe for human use (EFSA, 2009, 2012). Abramsson and Ilbäck (2013) using a flow-cytometric based micronucleus assay *in vivo* reported that intra-peritoneal administration of allura red does not cause a depression in cell proliferation or an increase in the frequency of micronuclei in polychromated erthrocytic cells.

The authors tested the dyes up to doses of 2000 mg/kg b.w. as suggested by the preliminary acute toxicity carried out by Sasaki et al. (2002). They also justified the use of the intra-peritoneal route on the poor uptake of allura red from the intestine and suggested that this coupled with the possibility of microbial formation of substances with local cellular action might partly explain the toxicity seen in the report by Shimada et al. (2010).

Sunset yellow FCF

Food Yellow No.5, CAS No. 2783-94-0.

Chemical name: Disodium 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonate.

Early reports demonstrated the non-mutagenicity of sunset yellow on *E. coli*, four tester strains of *S. typhimurium* TA 1538, 1535, 100 and 98 strains with or without metabolic activation (Chung et al., 1981; JECFA, 1982; Wever et al., 1989). Haveland-Smith and Combes (1980), also tested the ability of twenty five dyes to induce mutations in a tryptophan-requiring *E. coli* strain (sensitive to base substitutions) and a histidine auxotroph of *S. typhimurium* strain TA1538 (specific for frameshifts). Sunset yellow was demonstrated to be non-mutagenic with or without metabolic activation.

In vivo testing

Sunset Yellow FCF did not induce DNA damage in any of the eight mouse organs-glandular stomach, colon, liver, kidney, urinary bladder, lung, brain, bone marrow-assessed by comet assay following a single oral dosing of the dye up to 2000 mg/kg (Sasaki et al., 2002). Similarly, Poul et al. (2009) did not find any statistical difference in the gut micronuclei assay of groups of mice that had received the dye up to 2000 mg/kg b.w and a control group. The metabolites were monitored in the faeces using HPLC and reported to be non-genotoxic as well. The results of these studies agrees with that of Wever et al. (1989) who did not find any mutagenicity or clastogenicity effects when the dye was administered to rodents by gavage.

Chronic/long-term carcinogenicity studies

A number of studies describing the administration of sunset yellow in doses of 0 to 2% in mice for periods of 52 to 80 weeks have reported no significant difference in the incidence of tumours when compared with appropriate control groups (Bonser et al., 1956; Gaunt et al., 1974; JECFA, 1982; EFSA, 2009).Similar long-term studies in rats, hamster and dogs have not detected any carcinogenic effects associated with the dye when administered up to 5% doses (JECFA, 1982; EFSA, 2009).

Erythrosine

Food Red No. 3, CAS No. 16423-68-0
Chemical Name: disodium 2-(2,4,5,7-tetraiodo-6-oxido-3-oxoxanthen-9-yl) benzoate

Gene mutation assay

The inability of erythrosine to induce gene mutation in *S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538 at concentrations of up to 10,000 µg/plate have been demonstrated. The dye or its metabolites are reportedly non-mutagenic (Auletta et al., 1977; Bonin and Baker, 1980; Ishidate et al., 1984; JECFA, 1986; EFSA, 2011). In another report, erythrosine was not only non-mutagenic in *S. typhimurium* strains TA 97a, TA 98, TA 100, TA 102 and TA 104 but also reduced the anti-mutagenic potential of benzopyrene, sodium azide and ethidium bromide (Lakdawalla and Netrawali, 1988).

However, the mutagenicity of erythrosine has been reported in the *Bacillus subtilis* multigene sporulation assay (Lakdawalla and Netrawali, 1988) and *S. cerevisiae* strains D7 and XV185-14C (Matula and Downie, 1984). The *in vitro* genotoxicity of erythrosine using the Comet and Cytokinesis-block micronucleus cytome (CBMN-Cyt) assays have been investigated (Chequer et al., 2012). HepG-2 cells, which have the ability to mimic phase I and II metabolic activities, were incubated with dye concentrations of 0.1, 0.2, 2.0, 10, 25, 50, or 70 µg/mL. Results of Comet assay after 4 h of incubation showed significant DNA damage at 50 and 70 µg/mL when compared with the control group. Similarly, in the CBMN-Cyt assay, erythrosine caused an increase in micronuclei frequency at all doses greater than 0.1 µg/ml.

In another study to determine the cytotoxicity and genotoxicity of erythrosine using V79 hamster lung cells, the dye was cytostatic at 200 µg/ml and toxic to at least 90% of the cells at 400 µg/ml. In excess of 300 µg/ml genotoxicity was reported although this was not dose-related (Rogers et al., 1988). Using the Comet assay to monitor 8 mouse organs, Sasaki et al. (2012), also reported that the dye, after only 3 h post administration, produced dose-related DNA damage in the glandular stomach, colon and urinary bladder in mice that had received a single oral dose of 10, 100 or 2000 mg/kg b. w. The lowest dose that caused significant damage in the stomach and colon was 100 mg/kg bw.

Erythrosine (at doses of 50, 100 and 200 mg/kg b.w. repeated after 24 h) did not increase the frequency of sister chromatid exchanges in peripheral blood lymphocytes in male B6C3F1 mice. No increase in the frequency of micronuclei in the bone marrow polychromatic erythrocytes or peripheral blood reticulocytes was also observed. The authors suggested that the lack of a clastogenic potential provides support for the non-genotoxic mechanism of the carcinogenicity of erythrosine (Zuno et al., 1994).

Cytogenetic evaluation

A high degree of cytotoxicity and cytostaticity has been reported at erythrosine doses of 2,4 and 8 mM when tested on human peripheral blood cells *in vitro* (Mpountoukas et al., 2010). In the same study, extensive

direct binding of the dye to calf thymus DNA was reported.

Long-term carcinogenicity

Long term carcinogenicity studies have demonstrated that there was no significant difference in the incidence of non-neoplastic lesions or malignant tumours observed in the control and treated groups of Charles River CD weanling rats of both genders that have been exposed *in utero* to the dye and subsequently fed erythrosine doses of 0.1, 0.5, or 1.0% for 30 months. In the high dose part of the same study, in which groups of mice were fed 0 or 4% erythrosine in their diet for 29 months following *in utero* exposure, no dose-related effects were found in blood chemistry parameters, physical behaviour, urinalysis or mortality. However, there was an average of 100% gain in thyroid weights among the treated males as well as a significant increase in the incidence of follicular adenoma of the thyroid in the treated males compared to the control (Borzelleca et al., 1987; EFSA, 2011). A similar study in mice has been conducted (Borzelleca and Hallagan, 1987).

In another study, no tumour was observed in groups of Osborne Mendel rats that received oral gavages (0, 0.5, 1.0, 2.0 or 5.0%) or sub-cutaneous injections (12 mg/rat) for 24 months (Hansen et al., 1973). No mortality or dose-related adverse effects were reported in a similar study where groups of three female and three male beagle dogs were fed 0, 0.5, 1.0, 2.0 or 5.0% for 2 years (Hansen et al., 1973).

Reproductive and developmental toxicity

Erythrosine does not cause damage to the foetus following maternal exposure to dye (Collins et al., 1993a, b; EFSA, 2011). A number of studies have also failed to establish significant dose-related adverse effects in the reproductive toxicity and post-partum development of F0 and F1 generation of rats that received erythrosine (Vorhees et al., 1983; JECFA, 1986; Borzelleca et al., 1987; Tanaka, 2001). Erythrosine has also been discovered to possess no psychotoxic potential in developing rats (Vorhees et al., 1983). Erythrosine, however, was adjudged to have potential toxic effects on the reproductive process in male adult mice causing a decrease in the epididymal sperm count and motility as well as an increased frequency in sperm abnormalities (Abdel-Aziz et al., 1997; Vivekanandhi et al., 2006).

Brilliant Blue FCF

CAS No. 3844-45-9, Food Blue No. 1, FD & C Blue. No.1

Chemical Name: Ethyl - [4 - [[4 - [ethyl -[(3 - sulfophenyl) methyl] amino] phenyl] - (2 - sulfophenyl) methylidene] - 1 - cyclohexa - 2, 5 - dienylidene] - [(3 - sulfophenyl) methyl] azanium.

Gene mutation assay

The non-mutagenicity of brilliant blue in various *Salmonella* strains with or without metabolic activation has been demonstrated in many studies (Brown et al., 1978; Bonin and Baker, 1980; Haveland-Smith and Combes, 1980; Ishidate et al., 1984).

In vivo assays

Brilliant Blue did not increase the frequency of micronuclei in the bone marrow of groups of mice that received intra-peritoneal doses of 0, 500, 1000 or 2000 mg/kg b.w. (EFSA, 2010). In another study that was not considered by the last EFSA re-evaluation of brilliant blue because of the inconsistent osmolality of cell culture medium used, the dye was reportedly genotoxic in the chromosome aberration assay of over 190 additives using Chinese hamster fibroblast cell line (Ishidate et al., 1984; EFSA, 2010). The colourant at doses up to 2000 mg/kg b.w. gave negative results in a genotoxicity assessment of 39 food additives by comet assay on eight mouse organs (Sasaki et al., 2002).

Long-term/carcinogenicity studies

In a lifetime/carcinogenicity study that involve F0 and F1 generation, dye doses of 0, 0.1, 1.0 or 2.0% were fed to groups of Charles River CD rats for a period of 116 weeks for the males and 111 weeks for the females. A no-observed-adverse-effect-level (NOAEL) of 2 and 1% was established for the male and female groups respectively. In the same study with CD mice fed up to 5% dye, no adverse effects was observed and the NOAEL was established at 5% for both genders (Borzelleca et al., 1990). In a study conducted before the OECD guidelines were formulated and publicized, dietary concentrations of brilliant Blue, 0.03, 0.3 or 3% when fed to groups of 30 rats for 75 weeks did not produce any dose-related adverse effects on the growth or mortality (Mannell et al, 1962). Similar results were seen with the sub-cutaneous injection (Mannell and Grice, 1964).

Preservatives

In addition to the use of colourants, other agents are also included in food and other consumer goods. The inclusions of preservatives in such products are meant to

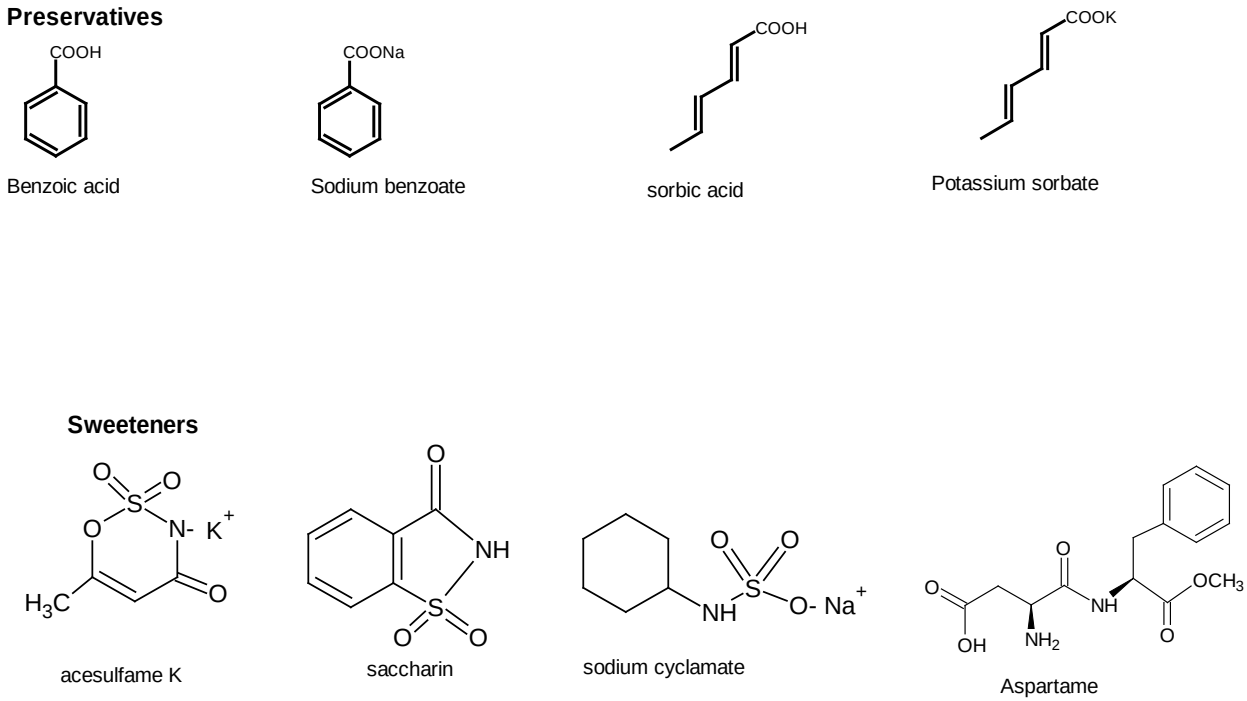


Figure 2. Some common food preservatives and additives.

serve the purpose of "warding off" microbial spoilage of such products. Safety concerns are also equally important in the use of these preservatives. The sections below give a review of some commonly used approved preservatives and the tests that have been carried out to determine their toxicity or otherwise. Figure 2 shows the chemical structures of the preservatives and sweeteners reviewed in this report.

Benzoic acid and sodium benzoate

The preservative could not induce umu gene expression in *S. typhimurium* TA 1535/pSK1002 which was defined as a 2-fold increase in the background activity of beta-galactosidase (Nakamura et al., 1987). Similar negative results were obtained in other studies with or without metabolic activation (Ishidate et al., 1984; Zeiger et al., 1988).

Cytogenetic assay

In a cytogenetic assay in which 50, 100, 200 and 500 µg/mL concentrations of the preservative were incubated with human lymphocytes, the additive, when compared with a negative control, caused dose-dependent increase in the frequency of micronuclei, sister chromatid exchange and chromosomal aberrations at all tested doses after 24 and 48 hours (Yılmaz et al., 2009). Chromatid breaks and sister chromatid unions were the most frequently reported aberrations in the study. After 48 h, 100, 200 and 500 µg/mL doses significantly reduce the mitotic index in a dose-related manner. This result agrees with another that concluded that sodium benzoate, potassium benzoate are clastogenic, mutagenic and cytotoxic to human lymphocytes *in vitro* (Zengin et al., 2011).

Benzoic acid was also found to increase chromosomal aberrations and decrease mitotic index in the root tips of Allium sativum after 24 and 48 h treatment with doses up to 500 mg/L (Yilmaz et al., 2008). Similar results were obtained with sodium benzoate (Njagi and Gopalan, 1982; Onyemaobi et al., 2012). Sodium benzoate was also positive in producing toxic effects using an in vitro screening with Chinese hamster cell line (Ishidate and Odashima, 1977; Ishidate et al., 1984).

In vivo assays

In a genotoxicity assessment of 39 food additives using comet assay on eight mouse organs, benzoic acid and sodium benzoate did not produce DNA damage in any of the organ at all tested doses (Sasaki et al., 2002).

Long-term carcinogenicity studies

The available studies on long-term carcinogenicity tests were conducted before the OECD guidelines and they are uniform in categorizing sodium benzoate as non-

carcinogens (Sodemoto and Enomoto, 1980; Toth, 1984). Despite some equivocal results obtained with *in vitro* chromosomal aberration assays, no positive genotoxic effects have been reported in an *in vivo* assay. In the light of this and the non-carcinogenicity in long-term assays, benzoic acid and sodium benzoate are classified as non-carcinogens (OECDS; SCCP, 2005).

Sorbic acid and sorbates

Oral administration of sorbic acid at doses up to 5000 mg/kg b. w. did not induce sister chromatid exchanges or increase in micronuclei in the bone marrow of mice. No DNA damage was observed when sorbic acid was incubated with human A549 cells or following intra-peritoneal treatment of rats with 500 to 1200 mg potassium sorbate/kg b. w. (Jung et al., 1992).

Other studies have reported the lack of mutagenic andcarcinogenic potential in both *in vitro* and *in vivo* models (Ishidate et al., 1984; Münzner et al., 1990; Walker, 1990; Schiffmann and Schlatter, 1992; Mamur et al., 2010). However, prolonged storage of sodium sorbate has reportedly yielded an oxidative degradation product, 4, 5-oxohexanoate which is mutagenic in the Ames test (Jung et al., 1992; Schiffmann and Schlatter, 1992) and may be partly responsible for the weak genotoxic results reported in cultured Chinese hamster V79 cells and wing spot test of *Drosophila* (Hasegawa et al., 1984; Schlatter et al., 1992).

The latter result emphasizes the need to consider the effect of metabolism on toxicities of the food colors and other additives. Metabolism of chemical compounds can either activate or deactivate a molecule. The end-results of exposure of a latent functional group in a chemical compound can lead potential toxicity.

Sweeteners

The development and use of sweeteners have been dogged by controversy and opinions are still divided as to the role they play in cancers especially of the brain and bladder, leukemia, lymphoma (Magnuson et al., 2007; Whitehouse et al., 2008; EFSA, 2013).

The use of artificial high-intensity sweetening agents in the food industry is increasingly becoming more popular. Their increased use is often tied to the promoted benefits of the products which include tooth friendliness, improved quality of life for patients of diabetes of various types and the allure of consumers enjoying their favourite 'unhealthy' drinks or snacks without the attendant problem of weight gain. This aggressive promotion has however not quietened many consumers who still maintain that artificial sweeteners are not safe and that their preference over beet sugar or cane sugar by the food industry has been solely for economic reasons. It is therefore necessary for the food industry to ensure consumer confidence by controlling the content of sweeteners in processed food and ensuring that the limit amount or concentration permissible in various types of food are not exceeded. Artificial sweeteners include those classified as the first generation-saccharin, cyclamate, aspartame and the new generation including acesulfame-K, sucralose, neotame. Different blends of the sweeteners are also used extensively in soft drinks (Weihrauch and Diehl, 2004). Another classification of artificial sweeteners grouped them into synthetic, semi-synthetic and natural. They comprise of a wide variety of organic molecules (example, carbohydrate derivatives, salts of organic acids, terpenoids and even proteins (Capitan-Vallvey et al., 2006).

Gene mutation assay

Aspartame, acesulfame-K and saccharin were found to be non-mutagenic in *S. typhimurium* strains T 97a and T 100a study (Bandyopadhyay et al., 2008). This study however is not compliant with the OECD guideline that requires full testing with five strains including: *S. typhimurium* TA1535, *S. typhimurium* TA1537 or TA97 or TA97a, and *S. typhimurium* TA98, *S. typhimurium* TA100 and *E. coli* WP2 uvrA, or *E. coli* WP2 uvrA (pKM101), or *S. typhimurium* TA102(OECD, 1997). Another study did not find any genotoxic potential when aspartame, acesulfame K, cyclamate, saccharin or sucralose were tested on hepatocytes from F344 and Sprague-Dawley male rats (Jeffrey and Williams, 2000).

In vivo assays

Chromosomal aberration assays in bone marrow of Swiss albino mice that had been fed blends of aspartame and acesulfame-K did not show any genotoxic activity (Mukhopadhyay et al., 2000). Contradictorily, acesulfame K at dose below the recommended no-toxic-effects amounts showed positive clastogenic potentials in a similar study (Mukherjee and Chakrabarti, 1997). Comet assay in eight organs of mice that have been fed the sweeteners showed that aspartame and acesulfame K lacked genotoxic potentials. However, sodium cyclamate produced significant DNA damage in the glandular stomach, colon, kidney and urinary bladder. Saccharin and sucralose were also genotoxic (Sasaki et al., 2002).

Long-term carcinogenicity studies

Contrary to public suspicions, there is no credible link between aspartame, saccharin or cyclamate and cancer. A number of long-term animal studies and epidemiological studies in human population have not shown that the use of these additives increases the risk of developing cancer in later life (Weihrauch and Diehl,

2004; Magnuson et al., 2007; EFSA, 2013).

However, two studies that reported tumourigenic potential generated a lot of media interest and ultimately led to withdrawal of saccharin in Canada and a label warning on all food containing saccharin (Squire, 1985; Taylor et al., 1980). Taylor et al. (1980), reported a significant increase in the incidence of neoplasms in F1 generation of male Charles River CD rats that received a 7.5% dietary concentration of saccharin. A subsequent report by Squire (1985) also found sodium saccharin related increase in the incidence of urinary bladder hyperplasia.

Saccharin would later be de-listed as a carcinogen following a study that showed that sodium ascorbate like most sodium salts (sodium saccharin inclusive) produces cytotoxic calcium phosphate containing crystals in the male rats. This important step in carcinogenicity is specific in rats because of a combined effect of high urine protein levels and the resultant high urine osmolality at levels that are not observed in humans (Cohen et al., 1998).

Role of metabolism in carcinogenicity

Mutagens that cause damage by interacting directly with DNA are categorized as genotoxic. The theoretical considerations that have been considered in explaining their mechanism of toxicity include DNA intercalation in between base pairs (Hendry et al., 2007) as well as metabolic activation following one or a combination of any of intestinal azo reduction, hepatic azo reduction, hepatic oxidation, liver microsomal enzymes activation (Moller and Wallin, 2000).

Metabolism plays important roles in the activity of xenobiotics in the body by producing highly reactive electrophiles that covalently bind to DNA and proteins (Guengerich, 2001). The reduction of the azo linkage in azo dyes by the intestinal bacteria produces aromatic amines which are responsible for the toxicity, mutagenicity and carcinogenicity of these dyes (Chung et al., 1992; Chequer et al., 2011). Over 45 intestinal bacteria express azoreductases that metabolize 2-hydroxylazo naphthols to aromatic amines (Zimmermann et al., 1982; Rafii and Cerniglia, 1995; Moller and Wallin, 2000). Microsomal hepatic azoreduction have also been reported with the food/drug additive amaranth. In a study, the activity of the azoreductase was greatly enhanced in mice by their pretreatment with the cytochrome P-450 inducers phenobarbital and 3-methylcholanthrene (Moller and Wallin, 2000).

Hepatic oxidation is mediated by cytochrome P-450 and the widely distributed perioxidase enzymes. Oxidation often produces highly unstable electrophilic free radicals that readily stabilize themselves by alkylating DNA, thereby causing disruption. Inhibition of Human CYP3A4 and P-glycoprotein by superoxide radicals generated from xanthene colourants have been reported (Mizutani, 2009). Many genotoxic substances have also been reported to act *via* direct DNA and/or protein adducts formation (Adegoke et al., 2012a; Adegoke et al., 2012b).

However, a large number of mutagens that do not directly interfere with DNA integrity have also been implicated in human carcinogenicity. Such substances, classified as non-genotoxic, act by effecting epigenetic changes. Epigenetics is defined as ‘’the heritable changes in the activity of gene expression without alteration of DNA sequences, which has been linked to many human diseases, including cancer. DNA methylation and histone modifications are well-known epigenetic changes that can lead to gene activation or inactivation” (Kawasaki and Abe, 2012). Such changes are transmitted through mitosis and meiosis processes and always occur with the preservation of the original DNA sequence (Brait and Sidransky, 2011; Kawasaki and Abe, 2012).

A number of mechanisms have been proposed for the toxicity of non-genotoxic carcinogens and these include peroxisome proliferation, aryl hydrocarbon receptor (AhR) binding, inhibition of gap junctional intercellular communication, oxidative stress, alteration of DNA methylation, endocrine disruption and generative cell proliferation (Kristensen et al., 2009; Serafimova et al., 2010).

The categorization of mutagens is not always distinct since they can act by a combination of mechanisms. Also, gene mutations are only partly responsible for tumour development as cancer is caused by a combination of factors (Sarasin, 2003; Sugimura et al., 1992). For example, *p*-chloroaniline is regarded as a genotoxic non-carcinogen that produces detectable genotoxic responses but not in sufficient levels as to initiate tumourigenesis in a long term bioassay (Kirkland and Speit, 2008). Similarly, a number of cellular damages like DNA strand breakage are repaired by the cells while some genotoxic processes do not occur with sufficient degree to lead to inheritable changes. This therefore requires that genotoxicity tests must not only be sensitive (able to give positive results with carcinogens) but also specific, that is, able to give negative results with non-carcinogens.

Limitations and challenges of genotoxicity assessment

Appropriate In vivo follow-up assay of genotoxicity

In addition to the recommended genotoxicity tests (FDA, 1993), there has been increasing calls for the inclusion of an appropriate *in vivo* genotoxicity test following a positive result in the *in vitro* assay. Although, the micronucleus assay has been conventionally used (Heddle et al., 1983; Heddle et al., 1991; Kirsch-Volders

et al., 2011), other tests such as those based on DNA adduct formation (Dybing et al., 1984), *in vivo* chromosomal aberration (You et al., 1993), transgenic mutation (Heddle et al., 2000; Lambert et al., 2005) and comet assay (Tice et al., 2000; Kumaravel and Jha, 2006; Olive and Banáth, 2006) have also been routinely employed in the past as *in vivo* follow up assay when equivocal or inconclusive results are obtained in the *in vitro* assay. Kawaguchi et al. (2010), reported an identical sensitivity of the micronucleus test and the comet assay in detecting the studied mutagens but were quick to point out that the power of the comet assay to detect a low level of genotoxic potential can be superior to that of micronucleus test by the inclusion of using DNA re-synthesis inhibitors.

In another study to compare the ability of these follow up tests to detect 67 known carcinogens that were missed in the traditional micronucleus test, transgenic assay detected about 50%, UDS about 20%, while the comet assay detected up to 90%. The comet assay also showed a high specificity giving negative results with non-carcinogen with an accuracy of up to 78% (Kirkland and Speit, 2008). When compared to other genotoxicity assessment methods, the comet assay is flexible, requiring only a small amount of sample and cells (Tice et al., 2000). It has also been most adaptable for the detection of various forms of DNA damage including double strand breaks, cross DNA damage and apoptosis (Olive and Banáth, 2006; Speit and Hartmann, 2006).

The comet assay has also been employed in multi-end point assay (Bowen et al., 2011) as it can be conducted using cells from virtually all organs (Kirkland and Speit, 2008). This is very useful as some chemicals may not require liver activation and may produce tumours in the tissue of first contact. It may therefore be necessary to assess DNA damage on the gastrointestinal tract for an oral drug, the rectum for a suppository or the skin for a topical preparation. However, the comet assay has some limitations as well with the most important of these being the overestimation of genotoxicity as DNA damages like strand damage, may ultimately be repaired or lead to cell death and not necessarily tumour (Kirkland and Speit, 2008).

There is also the need for the standardization of the methodologies of the comet assay to make it acceptable to all international regulatory agencies. Similarly, more studies are required to populate and validate comet assay data especially those obtained with organs other than the liver (Burlinson et al., 2007; Lovell and Pa, 2008).

Specificity

While the battery of tests described above remain the current recommendations for the genotoxicity testing of pharmaceuticals (ICH, 1997), their specificity has been questioned (Kirkland and Speit, 2008; Mahadevan et al., 2011). The test sets are particularly poor in detecting chlorinated substances and produce false negative results with a large number of carcinogens with non-genotoxic mechanisms (Mahadevan et al., 2011). Quantitative Structure-Activity Relationships (QSARs) predictive toxicology in recent times has been used not only in molecular design but also to clarify contradicting genotoxicity results (Benz et al., 2007; Contrera et al., 2007; Keshavarz et al., 2012).

Cytotoxicity

Meaningful assessment of genotoxicity can only be made after taking account for any confounding effects of cytotoxicity. A number of methods have been used to assess metabolically viable cells and these include dye exclusion technique using trypan blue (Adegoke et al., 2012), dual dye viability assay, histopathology and neutral diffusion assay (Tice et al., 2000). Although, there is no consensus yet as to the extent of cell viability that is acceptable in the comet assay, 70 to 80% cell viability is generally acceptable. In recent times, however, the abilities of the living cells to take up some dyes to the exclusion of dead cells have been used as index of cytotoxicity. In this regards, tests such as Evans blue staining, acridine orange and similar tests are currently used. Some other tests that measure metabolic activities are also prominently utilized to determine cell viability and detect if agents are cytotoxic or otherwise.

Overview of current toxicity status

This review has attempted to chronicle the various tests and reports that are available in literature on the toxicity profiles of approved food colours and additives. The literature is increasing daily on the assessment of the status of safety (or otherwise) of these agents. The fact that the agents are consumer goods gives them the tendency to be exposed to public scrutiny and assessments.

One concern about regulations available is that there are no uniform guidelines across the regional and worldwide agencies on the exact status of the agents. Food colourants and additives that are acceptable in the USA through FDA legislation may not be particularly acceptable in the EU countries or their uses are stringently controlled. Similar scenario of lack of uniformity also occurs in Asian countries. These call for the design of appropriate harmonization on the list of approved colourants and additives. One would imagine that if there is harmonization of test requirements for the assessment of safety status then there should also be uniform agreement on the number and type of these agents acceptable all over the world.One other concern that will require urgent attention is that of having internationally acceptable *admissible daily intake* (ADI).

The ADIs of most agents vary across the different regions of the world and this is a great concern. A worldwide acceptable tolerance limits in terms of consumption should be advised. There is a need to go beyond daily allowable intake. The argument that majority of these additives are water soluble, and hence readily excreted may not necessarily account for the lack of information on effects of cumulative consumption of these agents.

Another major consideration that would need urgent attention is the extrapolation of in vitro toxicological data from isolated human and mammalian cells to real practical use settings. There are so many limitations to the use of isolated cells and handling of these compounds may not sufficiently correlate with *in vivo* outcomes. Herein is the real concern in getting to know the appropriate tests that would adequately mimic the conditions *in vivo*. The use of whole animals and their body organs merely approximates to the biological outcomes that are anticipated upon ingestion of these additives on long term use. Indeed, many long-term use experiments in lower animals cannot provide adequate data for meaningful extrapolation to humans. The effects of carrying over residuals of approved colours and additives from animal sources upon consumption of animals and their products are other areas that will also require adequate attention in the near future.

CONCLUSION

The possibility of toxicity amongst such varied chemical structures means all new chemical entities are to be regarded as toxic until their safety is demonstrated. The choice of a particular method for evaluating a compound can therefore not be based on its chemistry alone or expected mechanism of action. Also, because of the often contradicting results and insufficient data to conclusively categorize many routinely used substances as safe or carcinogenic, there is need for constant evaluation of new chemical entities and existing ones as well.

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Conflicts of interest

The authors have none to declare.

REFERENCES

Abdel-Aziz AH, Shouman SA, Attia AS, Saad SF (1997). A study on the reproductive toxicity of erythrosine in male mice. Pharmacol. Res. 35(5):457-462.

Abramsson-Zetterberg L, Ilbäck NG (2013). The synthetic food colouring agent Allura Red AC (E129) is not genotoxic in a flow cytometry-based micronucleus assay in vivo. Food Chem. Toxicol. 59:86-89.

Adegoke OA, Ghosh M, Mukherjee A (2012b). Spectrophotometric and thermodynamic studies of the interactions of 4-carboxyl-2,6-dinitrophenylazohydroxynaphthalenes with bovine serum albumin. Spectrochim. Acta A Mol. Biomol. Spectrosc. 96:1038-1046.

Adegoke OA, Kyu JK, Mukherjee A (2012a). In vitro genotoxicity evaluation of 4-carboxyl-2,6-dinitrophenylazohydroxynaphthalenes using human lymphocytes. Food Chem. Toxicol. 50(3-4):936-941.

Auletta AE, Kuzava JM, Parmar AS (1977). Lack of mutagenic activity of a series of food dyes for Salmonella typhimurium. Mutat. Res. 56:203.

Bandyopadhyay A, Ghoshal S, Mukherjee A (2008). Genotoxicity testing of low-calorie sweeteners: aspartame, acesulfame-K, and saccharin. Drug Chem. Toxicol. 31(4):447-457.

Benz DR, Matthews EJ, Kruhlak NL, Frid AA, Minnier BL, Contrera JF (2007). Toxicological and clinical computational analysis by the Informatics and Computational Safety Analysis Staff of the US FDA/CDER. AATEX 14, Special Issue, 463-467, Proc. 6th World Congress on Alternatives & Animal Use in the Life Sciences, August 21-25, 2007, Tokyo, Japan. Available at: http://www.asas.or.jp/jsaae_old/zasshi/WC6_PC/paper463.pdf

Bonin AM, Baker RS (1980). Mutagenicity testing of some approved food additives with Salmonella microsome assay. Food Technol. Aust. 32(12):608-611.

Bonser GM, Clayson DB, Jull JW (1956). The induction of tumours of the subcutaneous tissues, liver and intestine in the mouse by certain dyestuffs and their intermediates. Br. J. Cancer 10(4):653-667.

Borzelleca JF, Capen CC, Hallagan JB (1987). Lifetime toxicity/carcinogenicity study of FD & C Red No. 3 (erythrosine) in rats. Food Chem. Toxicol. 25(10):723-733.

Borzelleca JF, Depukat K, Hallagan JB (1990). Lifetime toxicity/carcinogenicity studies of FD & C Blue No. 1 (brilliant blue FCF) in rats and mice. Food Chem. Toxicol, 28(4):221-234.

Borzelleca JF, Hallagan JB (1987). Lifetime toxicity/carcinogenicity study of FD & C Red No. 3 (erythrosine) in mice. Food Chem. Toxicol. 25(10):735-737.

Bowen DE, Whitwell JH, Lillford L, HendersonD, Kidd D, Mc Garry S, Kirkland DJ (2011). Evaluation of a multi-endpoint assay in rats, combining the bone-marrow micronucleus test, the Comet assay and the flow-cytometric peripheral blood micronucleus test. Mutat. Res. 722:7-19.

Brait M, Sidransky D (2011). Cancer epigenetics: above and beyond. Toxicol. Mech. Methods 21(4):275-288.

Brambilla G, Martelli A (2009). Update on genotoxicity and carcinogenicity testing of 472 marketed pharmaceuticals. Mutat. Res. 681(2-3):209-229.

Brown JP, Roehm GW, Brown RJ (1978). Mutagenicity testing of certified food colors and related azo, xanthene and triphenylmethane dyes with the Salmonella/microsome system. Mutat. Res. 56(3):249-271.

Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Hartmann A (2007). Fourth International Workgroup on Genotoxicity testing:Results of the *in vivo* Comet assay workgroup. Mutat. Res. 627:31-35.

Capitan-Vallvey LF, Valencia MC, Nicolas EA, Garcia- Jimenez JF (2006). Resolution of an intense sweetener mixture by use of a flow injection sensor with on-line solid-phase extraction. Application to saccharin and aspartame in sweets and drinks. Anal. Bioanal. Chem.

385:385-391.
Chequer F, Dorta D, Palma de Oliveira D (2011). Azo Dyes and their Metabolites: Does the Discharge of the Azo Dye into Water Bodies Represent Human and Ecological Risks? Advances in Treating Textile Effluent (P. P. Hauser Ed.) Croatia: InTech.
Chequer FM, Venancio VP, Bianchi ML, Antunes LM (2012). Genotoxic and mutagenic effects of erythrosine B, a xanthene food dye, on HepG2 cells. Food Chem. Toxicol. 50(10):3447-3451.
Chung K, Stevens S, Cerniglia C (1992). The reduction of azo dyes by the intestinal microflora. Crit. Rev. Microbiol. 18(3):175-190.
Chung KT, Fulk G, Andrews A (1981). Mutagenicity testing of some commonly used dyes. Appl. Environ. Microbiol. 42(4):641-648.
Cohen SM, Anderson TA, de Oliveira LM, Arnold LL (1998). Tumorigenicity of Sodium Ascorbate in Male Rats. Cancer Res. 58(12):2557-2561.
Collins TF, Black TN, Brown LH, Bulhack P (1990). Study of the teratogenic potential of FD & C Yellow No. 5 when given by gavage to rats. Food Chem. Toxicol. 28(12):821-827.
Collins TF, Black TN, O'Donnell MWJ, Bulhack P (1992). Study of the teratogenic potential of FD & C yellow No. 5 when given in drinking-water. Food Chem. Toxicol. 30(4):263-268.
Collins TF, Black TN, O'Donnell MWJ, Shackelford ME, Bulhack P (1993a). Teratogenic potential of FD & C red no. 3 when given in drinking water. Food Chem. Toxicol. 31(3):161-167.
Collins TF, Black TN, Ruggles DI (1993b). Teratogenic potential of FD&C Red No. 3 when given by gavage. Toxicol. Ind. Health 9(4):605-616.
Combes RD, Haveland-Smith RB (1982). A review of the genotoxicity of food, drug and cosmetic colours and other azo, triphenylmethane and xanthene dyes. Mutat. Res. 98(2):101-243.
Contrera JF, Kruhlak NL, Matthews EJ, Benz RD (2007). Comparison of MC4PC and MDL-QSAR rodent carcinogenicity predictions and the enhancement of predictive performance by combining QSAR models. Regul. Toxicol. Pharmacol. 49(3):172-182.
Das A, Mukherjee A (2004). Genotoxicity testing of food colours amaranth and tartrazine. Int. J. Hum. Gen. 4:277-280.
Dybing E, Holme JA, Perry GW, Søderlund EJ, Dahlin DC, Nelson SD (1984). Genotoxicity studies with paracetamol. Mutat. Res.138(1):21-32.
EFSA (2009a). EFSA Panel on Food Additives and Nutrient Sources added to Food; Scientific Opinion on the re-evaluation of Tartrazine (E 102) as a food additive. EFSA 7:1331-1383.
EFSA (2009b). EFSA Panel on Food Additives and Nutrient Sources added to Food: Scientific Opinion on the re-evaluation of Allura Red AC (E 129) as a food additive. EFSA 7(11):1327-1366.
EFSA (2009c). EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS): Scientific Opinion on the re-evaluation of Sunset Yellow FCF (E 110) as a food additive. EFSA 7(11):1330-1374.
EFSA (2010). EFSA Panel on Food Additives and Nutrient Sources added to Food:Scientific Opinion on the re-evaluation of Brilliant Blue FCF (E 133) as a food additive. EFSA 8(11):1853-1889.
EFSA (2011). EFSA Panel on Food Additives and Nutrient Sources added to Food; Scientific Opinion on the re-evaluation of Erythrosine (E 127) as a food additive. EFSA 9(1):1854-1900.
EFSA (2012). Scientific Opinion on the safety and efficacy of Allura Red AC (E 129) in feed for cats and dogs. EFSA 10(5):2675-2689.
EFSA (2013). EFSA Panel on Food Additives and Nutrient Sources added to Food; Scientific Opinion on the re-evaluation of aspartame (E 951) as a food additive. EFSA 11(12):3496-3759.
Elhkim MO, Heraud F, Bemrah N, Gauchard F, Lorino T, Lambre C, Poul JM (2007). New considerations regarding the risk assessment on Tartrazine An update toxicological assessment, intolerance reactions and maximum theoretical daily intake in France. Regul. Toxicol. Pharmacol. 47(3):308-316.
EU (2015). Current EU approved additives and their E Numbers. Available at: https://www.food.gov.uk/science/additives/enumberlist
FDA (1993). 1993 Draft Redbook II: Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food. Redbook II Draft Guidance. Available at: http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078717.htm
FDA (2015). Summary of Color Additives for Use in the United States in Foods, Drugs, Cosmetics, and Medical Devices. Available at: http://www.fda.gov/ForIndustry/ColorAdditives/ColorAdditiveInventories/ucm115641.htm
Fraumeni Jr J, Miller R (1972). Drug-induced cancer. J. Natl. Cancer Inst. 48(5):1267.
Gaunt IF, Mason PL, Grasso P, Kiss IS (1974). Long-term toxicity of Sunset Yellow FCF in mice. Food Cosmet. Toxicol. 12(1):1-9.
Golka K, Kopps S, Myslak Z (2004). Carcinogenicity of azo colorants: influence of solubility and bioavailability. Toxicol. lett. 151(1):203-210.
Guengerich FP (2001). Forging the links between metabolism and carcinogenesis. Mutat. Res. 488:195-209.
Güngörmüş C, Kılıç A (2012). The Safety Assessment of Food Additives by Reproductive and Developmental Toxicity Studies. Food Additive, Hacettepe University/Department of Biology, Ankara Turkey. Available at: http://www.intechopen.com/books/food-additive/the-safety-assessment-of-food-additives-by-reproductive-and-developmental-toxicity-studies
Hansen WH, Zwickey RE, Brouwer JB, Fitzhugh OG (1973). Long-term toxicity studies of erythrosine. I. Effects in rats and dogs. Food Cosmet. Toxicol. 11(4):527-534.
Hasegawa M, Nishi Y, Ohkawa Y, Inui N (1984). Effects of sorbic acid and its salts on chromosome aberrations, sister chromatid exchanges and gene mutations in cultured Chinese hamster cells. Food Chem. Toxicol. 22(7):501-507.
Haveland-Smith RB, Combes RD (1980). Screening of food dyes for genotoxic activity. Food Cosmet. Toxicol. 18(3):215-221.
Heddle JA, Cimino MC, Hayashi M, Romagna F, Shelby MD, Tucker JD, Mac Gregor JT (1991). Micronuclei as an index of cytogenetic damage: past, present, and future. Environ. Mol. Mutagen. 18(4):277-291.
Heddle JA, Dean S, Nohmi T, Boerrigter M, Casciano D, Douglas GR, Martus H (2000). *In vivo* transgenic mutation assays. Environ. Mol. Mutagen. 35(3):253-259.
Heddle JA, Hite M, Kirkhart B, Mavournin K, MacGregor J, Newell GW, Salamone MF (1983). The induction of micronuclei as a measure of genotoxicity: a report of the US Environmental Protection Agency Gene-Tox Program. Mutat. Res.123(1):61-118.
Hendry LB, Mahesh VB, Bransome Jr ED, Ewing DE (2007). Small molecule intercalation with double stranded DNA- Implications for normal gene regulation and for predicting the biological efficacy and genotoxicity of drugs and other chemic. Mutat. Res. 623:53-71.
Himri I, Bellahcen S, Souna F, BelmakkiF, Aziz M, Bnouham M, Saalaoui E (2011). A 90-day oral toxicity study of tartrazine, a synthetic food dye, in wistar rats. Int. J. Pharm. Pharm. Sci. 3(Suppl 3):159-169.
ICH (1997). Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals S2B Step 4 version International Conference on Harmonization (ICH) of technical requirements for registration of Pharmaceuticals for human use.
Ishidate Jr M, Odashima S (1977). Chromosome tests with 134 compounds on Chinese hamster cells in vitro--a screening for chemical carcinogens. Mutat. Res. 48(3-4):337-353.
Ishidate Jr M, Sofuni T, Yoshikawa K, Hayashi M, Nohmi T, Sawada M, Matsuoka A (1984). Primary mutagenicity screening of food additives currently used in Japan. Food Chem. Toxicol. 22(8):623-636.
Jabeen HS, ur Rahman S, Mahmood S, Anwer S (2013). Genotoxicity assessment of amaranth and allura red using Saccharomyces cerevisiae. Bull. Environ. Contam. Toxicol. 90(1):22-26.
JECFA (1982). 26th Report of the Joint FAO/WHO Expert Committee on Food Additives. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 17.
JECFA (1986). Toxicological evaluation of certain food additives and contaminants. 30th Report. WHO Food Additives Series.
Jeffrey AM, Williams GM (2000). Lack of DNA-damaging activity of five non-nutritive sweeteners in the rat hepatocyte/DNA repair assay. Food Chem. Toxicol. 38(4):335-338.
Jung R, Cojocel C, Muller W, Bottger D, Luck E (1992). Evaluation of the genotoxic potential of sorbic acid and potassium sorbate. Food Chem. Toxicol. 30(1):1-7.
Kamel MM, El-lethey HS (2011). The potential health hazard of

tartrazine and levels of hyperactivity, anxiety-like symptoms, depression and anti-social behaviour in rats. J. Am. Sci. 7(6):1211-1218.
Kawaguchi S, Nakamura T, Yamamoto A, Honda G, Sasaki Y (2010). Is the comet assay a sensitive procedure for detecting genotoxicity? J. Nucleic Acids 2010:541050.
Kawasaki H, Abe H (2012). Epigenetics in cancer and inflammation. Personal. Med. Universe 1:7-12.
Keshavarz MH, Gharagheizi F, Shokrolahi A, Zakinejad S (2012). Accurate prediction of the toxicity of benzoic acid compounds in mice via oral without using any computer codes. J. Hazard. Mater. 237–238:79-101.
Kirkland K, Speit G (2008). Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing *in vivo*. Mutat. Res. 654: 114-132.
Kirsch-Volders M, Decordier I, Elhajouji A, Plas G, Aardema M, Fenech M (2011). In vitro genotoxicity testing using the micronucleus assay in cell lines, human lymphocytes and 3D human skin models. Mutagenesis 26(1):177-184.
Kristensen LS, Nielsen HM, Hansen LL (2009). cancer and epigenetics. Eur. J. Pharmacol. 625:131-142.
Kumaravel TS, Jha AN (2006). Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. Mutat Res. 605(1):7-16.
Lakdawalla AA, Netrawali MS (1988a). Mutagenicity, comutagenicity, and antimutagenicity of erythrosine (FD and C red 3), a food dye, in the Ames/Salmonella assay. Mutat. Res. 204(2):131-139.
Lakdawalla AA, Netrawali MS (1988b). Erythrosine, a permitted food dye, is mutagenic in the Bacillus subtilis multigene sporulation assay. Mutat. Res. 206(2):171-176.
Lambert IB, Singer TM, Boucher SE, Douglas GR (2005). Detailed review of transgenic rodent mutation assays. Mutat. Res. 590(1):1-280.
Leconinte P, Lesca P (1978). Absence of activity of amaranth (FD & C red no. 2) in the Salmonella/microsome mutagenicity test. Food Cosmet. Toxicol. 16(1): 89-90.
Li Y, Wei H, Liu R (2014). A probe to study the toxic interaction of tartrazine with bovine hemoglobin at the molecular level. Luminescence 29(2):195-200.
Lovell D, Pa OT (2008). Statistical issues in the use of the comet assay. Mutagenesis 23(3): 171-182.
Maekawa A, MatsuokaC, Onodera H, TanigawaH, Furuta K, Kanno J, Ogiu T (1987). Lack of carcinogenicity of tartrazine (FD & C Yellow No. 5) in the F344 rat. Food Chem. Toxicol. 25(12): 891-896.
Magnuson BA, Burdock GA, Doull J, Kroes RM, Marsh GM, Pariza MW, Williams GM (2007). Aspartame: a safety evaluation based on current use levels, regulations, and toxicological and epidemiological studies. CRC Crit. Rev. Toxicol 37(8):629-727.
Mahadevan B, Snyder RD, Waters MD, Benz RD, Kemper RA, Tice RR (2011). Genetic toxicology in the 21st century: Reflections and future directions. Environ. Mol. Mutagen. 52(5): 339-354.
Mamur S, Yüzbaşıoğlu D, Ünal F, Yılmaz S (2010). Does potassium sorbate induce genotoxic or mutagenic effects in lymphocytes? Toxicol. *In vitro* 24(3):790-794.
Mannell WA, Grice HC (1964). Chronic toxicity of brilliant blue FCF, blue VRS, and green S in rats. J. Pharm. Pharmacol. 16(1):56-59.
Mannell WA, Grice HC, Allmark MG (1962). Chronic Toxicity Studies on Food Colours: V. Observations on the Toxicity of Brilliant Blue FCF, Guinea Green B and Benzyl Violet 4B in Rats. J. Pharm. Pharmacol. 14(1):378-384.
Matula TI, Downie RH (1984). Genetic toxicity of erythrosine in yeast. Mutat. Res. 138(2-3):153-156.
Mehedi N, Ainad-Tabet S, Mokrane N, Addou S, Zaoui C, Kheroua O, Saidi D (2009). Reproductive Toxicology of Tartrazine (FD and C Yellow No. 5) in Swiss Albino Mice. Am. J. Pharmacol. Toxicol. 4(4):130-135.
Mizutani T (2009). Toxicity of Xanthene Food Dyes by Inhibition of Human Drug-Metabolizing Enzymes in a Noncompetitive Manner. J. Environ. Public Health p 9.
Moller P, Wallin H (2000). Genotoxic hazards of azo pigments and other colorants related to 1-phenylazo-2-hydroxynaphthalene. Mutat Res. 462:13-30.
Mpountoukas P, Pantazaki A, Kostareli E, Christodoulou P, Kareli D, Poliliou S, Lialiaris T (2010). Cytogenetic evaluation and DNA interaction studies of the food colorants amaranth, erythrosine and tartrazine. Food Chem. Toxicol. 48(10):2934-2944.
Mukherjee A, Chakrabarti J (1997). *In vivo* cytogenetic studies on mice exposed to acesulfame-K--a non-nutritive sweetener. Food Chem. Toxicol. 35(12):1177-1179.
Mukhopadhyay M, Mukherjee A, Chakrabarti J (2000). In vivo cytogenetic studies on blends of aspartame and acesulfame-K. Food Chem. Toxicol. 38(1):75-77.
Münzner R, Guigas C, Renner HW (1990). Re-examination of potassium sorbate and sodium sorbate for possible genotoxic potential. Food Chem. Toxicol. 28(6):397-401.
Nakamura SI, Oda Y, Shimada T, Oki I, Sugimoto K (1987). SOS-inducing activity of chemical carcinogens and mutagens in Salmonella typhimurium TA1535/pSK1002: examination with 151 chemicals. Mutat. Res. 192(4):239-246.
Njagi GD, Gopalan HN (1982). Cytogenetic effects of the food preservatives--sodium benzoate and sodium sulphite on Vicia faba root meristems. Mutat. Res. 102(3):213-219.
OECD (1997). Test No. 471: Bacterial Reverse Mutation Test, OECD Guidelines for the Testing of Chemicals. Adopted 21/07/97. Available at: www.oecd.org/chemicalsafety/risk-assessment/1948418.pdf
OECDS/SIDS Initial Assessment Report (2001) Benzoates: Benzoic acid, Sodium Benzoate, Potassium Benzoate, Benzyl alcohol. Available at: www.inchem.org/documents/sids/sids/BENZOATES.pdf
Okada E, Fujiishi Y, Narumi K, Yasutake N, Ohyama W (2013). A four-day oral treatment regimen for simultaneous micronucleus analyses in the glandular stomach, colon, and bone marrow of rats. Mutat. Res. 758(1):87-94.
Olive PL, Banáth JP (2006). The comet assay: a method to measure DNA damage in individual cells. Nat. Protoc. 1(1):23-29.
Onyemaobi OI, Williams GO, Adekoya KO (2012). Cytogenetic Effects of two food preservatives, sodium metabisulphite and sodium benzoate on the root tips of *Allium cepa* Linn. Ife J. Sci. 14(1):155.
Poul M, Jarry G, Elhkim MO, Poul JM (2009). Lack of genotoxic effect of food dyes amaranth, sunset yellow and tartrazine and their metabolites in the gut micronucleus assay in mice. Food Chem. Toxicol. 47(2):443-448.
Rafii F, Cerniglia C (1995). Reduction of azo dyes and nitroaromatic compounds by bacterial enzymes from the human intestinal tract. Environ. Health Perspect. 103(Suppl 5):17-19.
Rall D (1975). Candidate drugs for carcinogenic property studies: procedures. J. Clin. Pharmacol. 15(1):1-4.
Rogers CG, Boyes BG, Matula TI, Heroux-Metcalf C, Clayson DB (1988). A multiple end-point approach to evaluation of cytotoxicity and genotoxicity of erythrosine (FD and C Red No. 3) in a V79 hepatocyte-mediated mutation assay. Mutat. Res. 205(1-4):415-423.
Sarasin A (2003). An overview of the mechanisms of mutagenesis and carcinogenesis. Mutat. Res. 544(2):99-106.
Sasaki YF, Sekihash K, Izumiyama F, Nishidate E,Saga A,Ishida K, Tsuda S (2000). The Comet Assay with Multiple Mouse Organs: Comparison of Comet Assay Results andCarcinogenicity with 208 Chemicals selected fromthe IARC Monographs and U.S. NTPCarcinogenicity Database. Crit. Rev. Toxicol. 30(6):629-799.
SCCP (2005). Scientific Committee on Consumer Products: Opinion on benzoic acid and sodium benzoate. sccp/0891/05. Available at: ec.europa.eu/health/ph_risk/committees/04_sccp/.../sccp_o_015.pdf
Schiffmann D, Schlatter J (1992). Genotoxicity and cell transformation studies with sorbates in Syrian hamster embryo fibroblasts. Food Chem. Toxicol. 30(8):669-672.
Schlatter J, Wurgler F, Kranzlin R, Maier P, Holliger E, Graf U (1992). The potential genotoxicity of sorbates: effects on cell cycle in vitro in V79 cells and somatic mutations in Drosophila. Food Chem. Toxicol. 30(10):843-851.
Serafimova R, Fuart-Gatnik M, Worth A (2010). Review of QSAR Models and Software for predicting carcinogenicity JRC Scientific and Technical Report. Luxembourg.
Shimada C, KanoK, Sasaki YF, Sato I, Tsuda S (2010). Differential colon DNA damage induced by azo food additives between rats and mice. J. Toxicol. Sci. 35(4):547-554.

Sodemoto Y, Enomoto M(1980). Report of carcinogenesis bioassay of sodium benzoate in rats: absence of carcinogenicity of sodium benzoate in rats. J. Environ. Pathol. Toxicol. 4(1):87-95.

Speit G, Hartmann A (2006). The Comet Assay: A Sensitive Genotoxicity Test for the Detection of DNA Damage and Repair. In: Henderson DS (Ed.), Methods in Molecular Biology: DNA Repair Protocols: Mammalian Systems, Second Edition, Totowa, NJ: Humana Press Inc. 314:275-286

Squire RA (1985). Histopathological evaluation of rat urinary bladders from the IRDC two-generation bioassay of sodium saccharin. Food Chem. Toxicol. 23(4-5):491-497.

Sugimura T, Terada M, Yokota J, Hirohashi S, Wakabayashi K (1992). Multiple genetic alterations in human carcinogenesis. Environ. Health Perspect. 98:5-12.

Tanaka T (2001). Reproductive and neurobehavioural toxicity study of erythrosine administered to mice in the diet. Food Chem. Toxicol. 39(5):447-454.

Taylor JM, Weinberger MA, Friedman L (1980). Chronic toxicity and carcinogenicity to the urinary bladder of sodium saccharin in the in utero-exposed rat. Toxicol. Appl. Pharmacol. 54(1):57-75.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Sasaki YF (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35(3):206-221.

Toth B (1984). Lack of Tumorigenicity of Sodium Benzoate in Mice. Toxicol. Sci. 4(3part1):494-496.

Tsuda S, Murakami M, Matsusaka N, Kano K, Taniguchi K, Sasaki YF (2001). DNA Damage Induced by Red Food Dyes Orally Administered to Pregnant and Male Mice. Toxicol. Sci. 61(1):92-99.

Vivekanandhi J, Devi CP, Jayaraman K, Raghavan L (2006). Effect of erythrosine on testicular function of mice. Toxicol. Int. 13(2):119-125.

Vorhees CV, Butcher RE, Brunner RL, Wootten V, Sobotka TJ (1983). A developmental toxicity and psychotoxicity evaluation of FD and C red dye# 3 (erythrosine) in rats. Arch. Toxicol. 53(4):253-264.

Walker R (1990). Toxicology of sorbic acid and sorbates. Food Addit. Contam. 7(5):671-676.

Weihrauch MR, Diehl V (2004). Artificial sweeteners--do they bear a carcinogenic risk? Ann. Oncol. 15(10):1460-1465.

Wever J, Munzner R, Renner HW (1989). Testing of sunset yellow and orange II for genotoxicity in different laboratory animal species. Environ. Mol. Mutagen. 13(3):271-276.

Whitehouse CR, Boullata J, McCauley LA (2008). The potential toxicity of artificial sweeteners. AAOHN J. 56(6):251-259; quiz 260-251.

Yilmaz S, Uenal F, Aksoy H, Yuezbasioglu D, Celik M (2008). Cytogenetic effects of citric acid and benzoic acid on Allium chromosomes. Fresenius Environ. Bull. 17(8 A):1029-1037.

Yılmaz S, Ünal F, Yüzbaşıoğlu D (2009). The *in vitro* genotoxicity of benzoic acid in human peripheral blood lymphocytes. Cytotechnology 60(1-3):55-61.

You Z, Brezzell M, Das S, Espadas-Torre M, Hooberman B, Sinsheimer J (1993). Ortho-Substituent effects on the *in vitro* and *in vivo* genotoxicity of benzidine derivatives. Mutat. Genet. 319(1):19-30.

Yu S, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K, Tsuda S (2002). The comet assay with 8 mouse organs results with 39 currently used food additives. Mutat. Res. 519:103-119.

Zeiger E, Anderson B, HaworthS, Lawlor T, Mortelmans K (1988). Salmonella mutagenicity tests: IV. Results from the testing of 300 chemicals. Environ. Mol. Mutagen. 11(S12):1-18.

Zengin N, Yüzbaşıoğlu D, Ünal F, Yılmaz S, Aksoy H (2011). The evaluation of the genotoxicity of two food preservatives: Sodium benzoate and potassium benzoate. Food Chem. Toxicol. 49(4):763-769.

Zimmermann T, Kulla H, Leisinger T (1982). Properties of Purified Orange II Azoreductase, the Enzyme Initiating Azo Dye Degradation by Pseudomonas KF46. Eur. J. Biochem. 129(1):197-203.

Zuno A, Marcon F, Leopardi P, Salvatore G, Carere A, Crebelli R (1994). An assessment of the in vivo clastogenicity of erythrosine. Food Chem. Toxicol. 32(2):159-163.

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