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# Simultaneous estimation of xanthine alkaloids (Theophylline, Theobromine and Caffeine) by High-Performance Liquid Chromatography

**Shruti Mourya[a], Ramesh Bodla[b,*], Ravikant Taurean[b], Akanksha Sharma[b]**

[a] *Quality Assurance Department, Delhi Institute of Pharmaceutical Science and Research, Delhi Pharmaceutical Sciences and Research University*, New Delhi, *India*
[b] *Pharmaceutical Chemistry Department, Delhi Institute of Pharmaceutical Science and Research, Delhi Pharmaceutical Sciences and Research University*, New Delhi, *India*

## Abstract

Methylxanthines are mainly a group of phytochemicals which are derived from purine base xanthine. These xanthines are obtained from plant as a result of secondary metabolism. There are various physiological actions that have been attributed to these derivatives in neurogenerative diseases, respiratory diseases, cancer and diabetes. The aim of this study is to develop a suitable qualitative and quantitative method for these xanthine derivatives. HPLC method is suitable for simultaneous estimation of methylxanthines, based on their physicochemical properties. Theobromine, Theophylline and Caffeine that belongs to alkaloids possess their economic effects. They have various stimulant effects on cardiovascular system, gastrointestinal system, respiratory system, central nervous system etc. that results in increased motivation to work, increased energy and increased alertness. High performance liquid chromatography is used for simultaneous determination of theophylline, theobromine and caffeine from different tea leaves.

**Keywords:** Xanthine alkaloids, Theobromine, Theophylline, Caffeine, Xanthines.

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*Corresponding author Tel.: +91-9711262640;
E-mail address: rameshbodla@gmail.com (R. Bodla).

## 1. Introduction

Xanthines are mostly produced by plants and animals. Xanthines are mainly involved in catabolic reactions of nucleotides and nucleic acids. Xanthine is a final product of purines through catabolism (1). Theophylline and caffeine are the most important xanthine alkaloids that are mostly found at large concentration in chocolates (2). Xanthine derivatives such as theophylline, theobromine and caffeine are widely found in food product, example: Tea, coffee, etc. Methylxanthines are produced by only limited number of plant species, i.e. tea (Camellia sinensis L.), coffee (coffea species), and cacao (theobroma cacao L.). Caffeine and theobromine are present at lower quantities in tea, coffee, cold beverages and chocolates. The more thoroughly studied methylxanthine is caffeine and it was first isolated from tea and coffee in 1820s. Theophylline and Theobromine are widely used as smooth muscle relaxant while caffeine has been studied for their various physiological effects on human beings. There are several other methods are also available for the extraction of caffeine. These are carbon dioxide extraction from green tea leaves, Microwave assisted extraction from green tea leaves and with mixtute of ethanol and water as a solvent (1, 3).

The use of theobromine and its related compunds in high concentration may cause nausea, gastritis, cancer, cardiac arrythmia and asthma. Food plays a major role in methylxanthine toxicity. Reversed phase high performance liquid chromatography (RP-HPLC) with UV-detector is one of the most important and common technique to analyse theobromine and its related compounds. This is just because of its effectiveness, speed and specificity (1, 4). Xanthine alkaloids such as theobromine, theophylline and caffeine are present in tissues of different woody species (5). Phenolic

compounds, in plants are an important part of the human diet, and they are considerable interest because of their antioxidant properties. The antioxidant activity of phenolic compounds depends on the structure, in a particular number and their positions of the hydroxyl groups and the nature of substitutions on the aromatic rings (6).

***Types of Tea leaves***

All Tea leaves are obtained from the plant *camellia sinensis* but there are various different varieties of tea leaves are also available with their own taste and aroma (7). Four types of tea are produced by the leaves of *Camellia sinensis Linn*.

(1) White Tea

(2) Green Tea

(3) Oolong Tea

(4) Black Tea

(5) Pu-Erh Tea

**White Tea-** White Tea is obtained from the plant *camelia sinensis*. White tea is least processed as compared to green which is non-fermented and oolong tea which is partly fermented (7) Famous white tea variety: SILVER NEEDLE (BAI HAO YIN ZHEN)

**Green Tea-** Green tea is obtained by withering, steaming, rolling and drying of tea leaves. Green tea undergoes least processing and mainly contains 80-90% catechins and 10% of flavanols (7).

*Famous Chinese variety: pi lo chun*

**Oolong Tea-** Oolong tea is partly fermented tea. It has fragrent flavour and sweet fruity aroma. The manufacturing process of oolong tea is same as green tea and oolong tea that's why it possess similar qualities as green tea and black tea.It has low level of caffeine (7).

*Famous oolong variety:* Dong Ding.

**Black Tea-** Black tea has additional processing steps which includes aeration and withering. It contains catechins (20-30%) and flavonoid (theaflavins and thearubigins 10% and total flavonoids is 50-60%. Black tea is most commonly used by human beings (7).

*Famous Chinese black tea: Yunnan.*

**Pu-Erh Tea-** Pu-Erh tea is totally fermented tea. It contains low level of caffeine. New studies found that Pu-Erh tea reduce the level of cholesterol (7).

**Biological Source**

It contains leaves and leaf buds of *Thea sinensis (Linn.)* belonging to family *Theaceae* (8).

**2. Xanthine Alkaloids**

Xanthine alkaloids is a fusion of an imidazole ring and pyrimidine rings. The group of xanthine alkaloids mainly contains caffeine, theobromine and theophylline (1, 9).

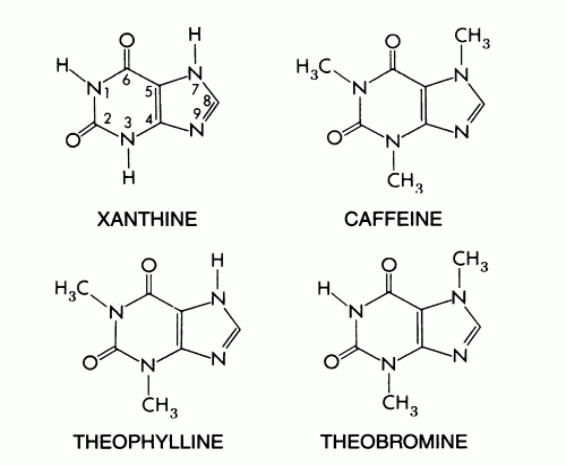


**Figure 1.** Xanthine Alkaloids

Xanthine is most commonly found in human tissues and fluids and in other organisms. Methylated xanthines of xanthines are called as methylxanthines. Methylxanthines are mainly heterocyclic organic compounds obtained from coupled pyrimidinedione and imidazole rings. Naturally occurring methylxanthines are caffeine(1,3,7-trimethylxanthine),Theophylline (1,3-dimethylxanthine), and theobromine (3,7-dimethyl xanthine). The example of more complex substituted methylxanthines are aminophylline (1,3-dimethyl-7H-purine-2,6-dione), Pentoxiphylline (1-(5-oxohexyl)-3,7-dimethylxanthine), and IBMX (3-isobutyl-1-methyl xanthine) (4, 10).

***Percentage of xanthine alkaloids***

There are mainly 13 orders of the plant kingdom in which 100 species of methylxanthines have been detected. Content of caffeine in coffee is to be about 0.4-2.4% dry weight. Content of caffeine in young leaves of *camellia sinensis, camellia assamica and camellia taliensis* is 2%-3% dry weight but it represents less than 0.02% in *camellia kissi* while the content of caffeine in tea(infusion) is in between 1.0% and 3.5% of the composition (1, 11).

Mainly caffeine being generally present in tea, some species of *camellia* accumulate theobromine instead of caffeine.

Tea and coffee beans naturally contains theophylline. Theobromine level is reported only 0.15-0.46% in different types of chocolates (1, 11).

***Natural sources***

Tea is mainly containing less amount of caffeine as compared to coffee. Tea is a more complex mixture of about 2000 chemical compounds including proteins, polysaccharides, minerals and trace elements, lignins, organic acids. Methylated xanthines are produced from purine nucleotides in plants (1, 12).

In the biosynthesis of methylxanthines, the initial substrate is xanthosine, which may be supplied by different pathways such as de novo purine biosynthesis (de novo pathway), adenine nucleotides degradation (AMP route), S-adenosyl methionine cycle (SAM route) and guanine nucleotides (GMP routes). Anabolism of caffeine is similar to the anabolism of other methylxanthines. Higher plants produced caffeine through a dominant pathway i.e. formation of xanthosine to 7-methylxanthine, to 7-methylxanthine, to theobromine, to caffeine pathway. Theobromine, theophylline and caffeine may also be produced by chemical synthesis.

There are many different techniques that have been used for the determination of methylated xanthines through many different sources and types. It is easy to find out various proposed protocols for the determination of methylxanthine (caffeine, theobromine and theophylline) from tea, food, beverages and even biological fluids (1).

There are various different kinds of extraction methods available that have been used to eliminate unwanted matrix interference in the determination of methylxanthines, including solid-phase exraction, liquid-liquid extraction and microwave assisted extraction. Water is used as a best solvent for the determination of methylxanthines because water is highly nonselective. There are some different solvents which is used for liquid extraction. These solvents includes chloroform, methanol, methylene chloride and n-hexane. These solvents are used for the extraction of methylxanthines from some natural plants (1, 12).

***Alkaloid Drugs from various plant sources***

**Table 1** Alkaloid drugs obtained from plant source and their activity (13).

| Alkaloid medicine | Plant | Activity | Product |
|---|---|---|---|
| **Atropine** | *Atropa Belladonna* | Antidote for Nerve gas poisoning | Abdominol, Espasmo, Protecor |
| **Ajmaline** | *Rauwolfia serpentine* | Antiarrythmic | Gilurytmal, Rauwopur |
| **Codeine** | *Papaver somniferum* | Analgesic(Painkiller) | Antituss, Codicaps, Tussipax |
| **Cocaine** | *Erythroxylum coca* | Local Anesthetic | Mainly used in highly regulated clinical environment. |
| **Colchicine** | *Colchicum autumnale* | Gout remedy | Colgout, Verban |
| **Caffeine** | *Coffea Arabica* | Central nerve system stimulant | Agevis, Thomapyrine, Vomex A |
| **Ephedrine** | *Ephedra sinica* | Antiasthmatics | Amidoyna, Bronchicum, Peripherin |
| **Ementine** | *Carapichea ipecacuanha* | Antiprozoal | Ipecac, Rectopyrine |
| **Morphine** | *Papaver somniferum* | Pain relief, Diarrhea | Duromorph, Spamofen |
| **Noscapine** | *Nicotiana tobacum* | Stimulant, nicotinic acetylcholine receptor agonist | Nicabate, Nicorette, stubit |
| **Vinca Alkaloids (Vincristine)** | *Vinca rosea* | Anticancer | Marqibo, Navelbine, oncovin |
| **Vinblastin** | *Catharanthus roseus* | Antitumor | Periblastine, velban, velsar |
| **Ceserpine** | *Rauwolfia serpentine* | Antihypertensive | Abicol, Briserin, sandril |
| **Pilocarpine** | *Pilocarpus pennatifolius* | Myotics | Salegen, vistacarpin |

***Antioxidant Properties of Green Tea***

The Antioxidants that are present in green tea (Catechins) are demonstrated to slow the growth of cancer cells, reduce the size of tumors, and decrease the adverse effects of chemotherapy. In asian countries green tea is consumed as plain brewed tea, but in western countries flavoured green tea is gained popularity and available in market. Green tea contains a high concentration of polyphenols (14). Green Tea leaves are converted into black tea leaves. Inspections have indicated that black tea contains minor amount of polyphenols that also reveals antioxidant behavior (15).

Tea has been extensively studied for its wide range of pharmacological benefits, including anti-diabetic, anti-oxidant, anti-cancer, and anti-microbial capabilities. Other benefits of green tea is leading to weight loss, promoting digestion and ingestion of fatty food and cholesterol (14, 15) Most famous Green-Tea based and foods for specified health uses (FOSHU) beverages are marketed in 500-555- and 300-600-mL bottles, respectively. Thus, the approximated EGCG contents per bottle of famous and FOSHU beverages are 30-80 and 50-145 mg, respectively (16).

## 3. Analytical Techniques

Various analytical techniques have been used for the analysis and identification of methylxanthines (Theobromine, theophylline and caffeine) such as capillary gas chromatography(GC), Gas chromatography -mass spectrometry (GC-MS), Spectrophotometry, capillary electrophoresis, Fourier transform-Raman spectrometry, Spectrofluorimetry and micellar electrokinetic electrophoresis (MEKC), voltametry, Radioimmuno assay (RIA),Thin layer chromatography (TLC), ion exchange chromatography and solid-phase ultraviolet sensing. Reversed phase High performance liquid chromatography (RP-HPLC) is the most common method of choice for the determination, identification and quantification of methylxanthines. Methylxanthines are separated through HPLC by the use of C-18 column and several solvents that are used as mobile phase in different combinations. Most commonly used solvents are water + Methanol/ Ethanol + acetic acid or water + acetonitrile. Effective separation has been achieved through either isocratic or gradient elution profiles. Most commonly used analytical techniques with HPLC method is coupled to mass spectrometry (1, 16).

### *Constituents of alkaloids in Tea*

The main alkaloid constituents in tea leaves is belongs to polyphenol group. It contains about 25-30% on a dry weight basis (16, 17).

There are mainly 6 group of compounds which is present as polyphenol in tea: Flavanols, Hydroxyl-4-flavanols, Anthocyanins, Flavones, Flavonols and Phenolic acids (16).

Major, important and characteristic polyphenols in tea are the flavanols in which catechins are predominant. The major catechins are: (-)-Epicatechins (EC), (-)-Epicatechin gallate (ECG), (-)-Epigallocatechin gallate (EGCG), (+)- Catechin (C), and (+)-Gallocatechin (GC). (17).

These compounds are mainly responsible for the bitterness, astringency and sweet after taste of tea beverages (17).

2%-4% of methyxanthines are mainly present as caffeine in tea and small amount of theobromine and theophylline is also present. Tea also contains vit.E, K, A and carbohydrates. Small amount of vit.B and vit.C is also present in tea. Amino acid is the main content of tea, accounting for 50% of the total amino acids (1). Chemical composition of green tea is complex: Proteins (15-20% dry weight), Amino acids (1-4% dry weight) just as glutamic acid, tryptophan, glycine, leucine, valine, arginine, aspartic acid, serine, lysine; Carbohydrates (5-7% dry weight) such as cellulose, pectins, glucose, fructose, sucrose; Lipids such as linoleic and linolenic acids, Vitamins (B,C,E); Bases such as caffeine and theophylline; trace elements (5% dry weights) like Ca, Mg, Mn, Ni, Se, Mo, Cu, Fe, and Na (18, 19).

**Minerals-** Tea contains about 4-5% of inorganic matter(Fluorine, nickel, potassium, aluminium, iodine, selenium and manganese) as mineral constituents (20).

### *Physiological effects*

The interesting fact is that, the methylxanthines produced significant biological effects. They possess low level of toxicity and they justified the attension to these compounds. The study describes their potential benefits related to several diseases (20).

Nervous system is the more obvious target of methylxanthines. Caffeine is used as an analgesic and it is used with some other analgesics such as paracetamol, ibuprofen or acetyl salicylic acid. Methylxanthines also possess psychostimulatory activity. Caffeine shows more health benefits in both animals and human studies (20).

Recently, the study of methylxanthines related to neurodegenerative diseases has gathered a considerable information. Regular uptake of caffeine or coffee has been related to the lower risk of alzheimer's disease and parkinson's diseases. The mechanism of adenosine receptor (A1 and A2A receptor) antagonism was suggested behind the neuroprotective effects of caffeine. However, the beneficial effects justified the protection against blood brain barrier dysfunction. It is cleared from the more recent animal and epidemiologic studies that there is a link between midlife caffeine consumption and lower disease incidence. Other than alzheimer's and parkinson's diseases, higher uptake of caffeine also showed some characteristics of machado-Joseph disease. Methylxanthines have also been used for respiratory diseases. Caffeine is used for the prematurity and apnea. The action of caffeine is used as a ventilator stimulant. Caffeine has been mostly assigned to antagonize the adenosine receptors in central respiratory centres. Inhibition of cAMP-dependent phosphodiesterase-4 also plays an important role in the neonatal carotid body. Theophylline was used for the treatment of asthma since 1920's. Theophylline is used in chronic obstructive pulmonary diseases (COPD). Methylxanthines also possess vasodilator effects and used to improve microcirculation of blood. These effects are commonly mediated by cAMP levels that are increased by the inhibition of phosphodiesterase activity. Methyl-xanthines also possess some benefits related to cardiovascular diseases and used for the treatment of angina syndrome and congestive heart failure. Methylxanthines reduces the risk of coronary heart disease and strock. In fact theobromine increases the level of high density lipoprotein (HDL) cholesterol and decreases the level of low density lipoprotein (LDL) cholesterol (1).

Research studies have shown that methylxanthines maximizes the obesity factor. Methylxanthines, namely theophylline and caffeine, have been shown diuretic and natriuretic effects (1, 5, 20).

### *Drawbacks and toxicity*

Nowadays the most common thing about methylxanthine consumption is related to prenatal exposure. There are several studies which are mostly conducted in animals and which revealed detrimental actions of methylxanthine during pregnancy. Human epidemiologic studies describes the effect of caffeine consumption at the time of pregnancy results in risk of

miscarriage and incidence of malformation. However, methylxanthine consumption should be limited in future mothers.

Another effect of methylxanthine is related to male fertility. Animal studies of methylxanthines (Theophylline, theobromine and caffeine) have been shown that the administration of theophylline, theobromine and caffeine may induce testicular atrophy and aspermatogenesis.

Modular consumption of caffeine is a safe habit. The aspects of higher caffeine uptake are gastrointestinal disturbances, insomnia, nervousness, headache, arrhythmia, nausea, tachycardia. The more extreme symptoms includes myopathy, hypokalemia, nausea, vomiting, diarrhoea and weight loss (1, 20).

**Table 2** Respective Pharmacological potencies of the naturally occurring Methylxanthines differing from more potent (+++) to less potent (+) (1, 20)

| Systematic effect | Caffeine | Theobromine | Theophylline |
|---|---|---|---|
| CNS Stimulation | +++ | + | ++ |
| Respiratory Stimulation | +++ | + | ++ |
| Diuresis | ++ | + | +++ |
| Coronary Dilation | + | ++ | +++ |
| Cardiac Stimulation | + | ++ | +++ |
| Skeletal Muscle Stimulation | +++ | + | ++ |
| Smooth muscle relaxation (Bronchodilation) | + | + | +++ |

Theophyllines possess stronger toxic effects than caffeine. Theophylline consumption at higher level can cause headache, nausea, vomiting, increased acid secretion and gastroesophageal reflux. Theobromine possess higher lethal doses (oral) than caffeine in human. It is also dangerous in other mammals (1, 20).

***Apparatus***

Vaishali Sharma et.al describes in their article that a merck hitachi HPLC system must be equipped with a vaccum degasser, a quaternary pump which is used to make gradients. This pump is programmable, thermostatic controlled column chamber, and a rheodyne injection valve is required with a 20µL sample loop for the study. The diode array detector was also used for the study. All the modules were controlled through a PC and a HPLC system window based software. A column C-18 (250×4.6mm, 5µ) is required and fitted with a suitable guard column (22). Acetonitrile or 0.1% orthophosphoric acid in water (w/v) was used as a mobile phase with a flow rate of 1mL/min. The temperature of column compartment is adjusted at $35^0$C (21).

***Analysis of Theobromine***

Amanda reges et.al studied in their review article and describes that the average amount of theobromine and caffeine that are obtained by caudle et al. (14) was 5.1mg/100g; the accuracy and precision were compared by Association of official Analytical chemistry (AOAC).The AOAC method is used for both internal standards and external standards. The additional standard method is more accurate and precise because it does not require any organic solvents, so that the solid contents of methyxanthine in chocolate may not be detected (21).

***Analysis of Theophylline***

Peter J. James described in their article that theophylline (1,3-dimethyxanthine) is effectively used for the treatment of asthma as a bronchodilator. It is also used in obstructive lung diseases. HPLC method has been developed for the rapid estimation of theophylline from various types of tea leaves. Recently theophylline at lower concentration has been shown their anti-inflammatory effects in asthma and chronic obstructive pulmonary disease (COPD).That's why theophylline is highly prescribed drug in asthma and COPD. Theophylline is a natural alkaloid which is present in green tea, black tea, coffee, and coca at different concentrations (21).

***Analysis of Caffeine***

Marcia S. Bispo et.al. and D. Komes et.al. studied that caffeine (1,3,5-trimethylxanthine) is present in various types of teas and it is most popular because of its pharmacological effects including stimulation of central nervous system (CNS),Gastrointestinal and respiratory system, smooth muscle relaxation, peripheral vasoconstriction, and myocardial stimulation. The amount of caffeine in tea leaves is 3.5%.Caffeine is bitter in taste and present in a crystalline form (22, 23).

***Analysis of Caffeine through HPLC***

Sample Preparation- D. Komes et.al. and Janna Erickson studied that tea bags containing green tea were weighed out (0.5 gm).Samples were extracted with 125mL Erlenmeyer flask in acetonitrile (60 mL) and distilled water (40 mL) for 1 hour with continuous stirring. Then solutions are then filtered and then diluted with 100mL of distilled water. Lipton tea which is in liquid form was sonicated and then 20mL portion is diluted in 50mL of distilled water. Then the sample solutions were fitered with a 0.45micrometer syringe filter (22).

According to the method, caffeine solution and filtered tea extract were injected into the HPLC system for analysis. Equipment includes varian pro star solvent delivery system 230 and a photodiode Array detector varian pro star 330 (varian, walnut Greek, USA) with a reversed phase column, C-18 (250 x 4.6 mm), 5 micrometer.

Caffeine can be determined by comparing the Retention times and spectral data with authentic standards. The analysis were repeated three times (23).

## 4. Results and Discussion

### *Elution method*

Different types of elution methods such as isocratic, gradient and combination elutions is used for the separation of biochemicals present in tea by HPLC. When total isocratic method was employed, a complete separation baseline for theophylline, epicatechin (EC), and epigallocatechin (EGC) could not be obtained. When total gradient method was employed, upward trailing in the baseline and initial level was obtained and baseline separation for theophylline, epigallocatechin (EGC), gallic acid (GC) could not be obtained just like isocratic method. When combination of isocratic and gradient method was employed, complete separation could be observed. Retension time of different compounds were achieved (23).

### *Mobile phase effects*

There are two mobile phases that were tested. One containing methanol or 0.1% orthophosphoric acid (w/v) in water and other one is containing acetonitrile or 0.1% orthophosphoric acid (w/v) in water. The first one mobile phase have been used for the separation of catechins from tea mixtures and other one mobile phase gave a complete separation of catechins, caffeine, gallic acid, thepbromine and theophylline. Similarly, complete separation was obtained by wang et.al. (2000) by employing mobile phase such as methanol or 0.1% orthophosphoric acid in water for the separation of tea catechins and caffeine but when they employed isocratic method for separation, the run time was very long (60 min.)

The separation should not be marked with methanol or 0.1% orthophosphoric acid system (23).

### *Absorption wavelength selection*

Different types of absorption wavelengths have been employed by different workers for the separation of catechins from tea, ranging from 210-280nm. All catechins, gallic acids (GA), and caffeine have been shown maximum absorbance at 210 nm and from 275-280 nm (23).

### *Column temperature effect*

Temparature of column is very important for the detection of biochemicals through HPLC system. The analysis was performed at different temperature such as 16°C, 30°C, 35°C, 40°C, 45°C for the detection of different biochemicals by the use of HPLC system. At lower temperature of the column, the chromatograms showed a baseline that merge theophylline and theobromine, and catechins and caffeine (23).

Beyond 35°C, the peak became very sharp but it is merged that makes the analysis of epicatechin (EC) and epigallocatechin gallate (EGCG), and theophylline and epigallocatechin (EGC). Different optimum chromate-graphic separations was achieved at 35°C for all biochemicals. As the temperature of column increased, retension time of biochemical decreased (23).

## 5. Conclusion

The main conclusion of this article is that all the Methylated xanthines are pharmacologically active compounds and they have been more or less noticeable part of human diet for centuries now. It is considerable that the advantage of methylxanthines may represent in human physiology and it is largely suppress detrimental effects. There are also some deleterious outcomes related to fertility and prenatal exposure. From the recent studies, consumption of methylxanthines in diet from many sources should still be consider safe (1). For the determination of methylxanthines a gradient method was developed in green tea and herbal tea. This method is rapid for routine analysis with short run times. By the use of this method theobromine, theophylline and caffeine were well separated. This method is simple, sensitive and accurate and can be appied to all kinds of tea (1, 10, 23).

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## Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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