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SPASMOLYTIC ACTIVITY OF CHLOROFORM FRACTION FROM *MORINDA MORINDOIDES* LEAVES (BAKER) MILNE-REDH. (RUBIACEAE) USED TO TREAT DIARRHOEA IN TRADITIONAL MEDICINE AND ITS ISOLATED CONSTITUENTS

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ABSTRACT
The present investigation reported the spasmolytic activities of aqueous extract, isolated flavonoids and iridoids form *Morinda morindoides* leaves which can explain in part, its use as antidiarrhoeal remedy in traditional medicine. Results from this evaluation activity revealed that chloroform soluble fraction, isolated flavonoids and iridoids induced significant acetylcholine (ACh) and depolarizing solution rich in KCl (DSR-KCl) inhibition of isolated guinea-pig ileum contractions by more than 65% at a tested concentration of 40 µg/ml in organ batch. The most active sample was chloroform soluble fraction producing 97.35% inhibition of ACh and 85.30±0.05% of DRS-KCl induced contractions of isolated guinea-pig ileum. Flavonoids chrysoeriol, luteolin, kaempferol and iridoids gaertneric acid, actylgaertneroside, epoxymethoxygaertneroside, dehydrogaertneroside, dehydromethoxygaertneroside, gartneric acid and methoxygaertneroside produced 70 < % inhibition < 80% and 65 < % inhibition < 76% of contractions induced by ACh and DSR-KCl respectively on isolated organ. These results partly suggested that aqueous extract of *M morindoides* can act as antidiarrhoeal agent by the spasmolytic activity of its flavonoids and iridoids.

KEYWORDS: *Morinda morindoides*, Rubiaceae, leaf, flavonoids, iridoids, spasmolytic activity, cytotoxicity.

INTRODUCTION
Diarrhea is a major health problem in children under 5 year olds mainly in developing countries. It is estimated 5-8 million deaths in infants and children each year below 5 year olds in these countries. In addition, according to WHO, about 5.8 to 7,1 million people death because of diarrhea infection (Ngo Teke et al., 2010; Yaganada et al., 2012; Balekar et al., 2014). It is characterized by an increase in frequency of bowel movements, wet stool and abdominal pains (Ngo Teke et al., 2010; Xuejong et al., 2014). Despite the efforts of many governments and international organizations to treat and cure this disease, its incidence still remains high (Yaganada et al., 2012). WHO has encouraged studies for treatment and prevention of diarrheal diseases using traditional medical practices and the use of oral rehydratation for treatment (Yadav et al., 2013; Balekar et al., 2014;).

Morinda morindoides (Baker) Milne-Redh. (Rubiaceae) (Synonym: *Gaertnera morindoides* Bak. or *Morinda confusa* Hutch.) commonly called in vernacular languages as Nkonga bululu in Tshiluba, Nkongo bololo or Nkama meso (literal traduction: plant to or with hundred eyes) in Lingala and Kikongo, Kilulu kundju in Swahili in Democratic Republic of Congo, is one of the most popular medicinal plants used daily in villages and towns in this country in often at home or in traditional medicine after to have consulted a tradipractioner. An aqueous decoction of fresh leaves, which is the typical traditional remedy is known by all people and does not require the presence of a tradipractioner for its preparation. It is used for the treatment of various illnesses such as diabetes, diarrhea, intestinal worms, rheumatism, amoebiasis, malaria and fever, infectious wounds, cutaneous eruptions, abdominal pains, constipation, hemorrhoids, rheumatism, gastralgia, icteria, blennorragia, dermatological diseases such as mycosis and scabies. It is also employed as oral tonic, stimulant of appetite and against general tiredness in children and adults (Kerharo and Adam, 1974, Ajanahoum el al, 1988; Kambu et al. 1990, 2009).

Previous studies on this medicinal plant part have reported some interesting biological activities related to some of traditional uses of *M. morindoides* leaf aqueous extract (decoction). These include the *in vitro* anticomplementary (Cimanga eta al., 1995b, 1197a,b, 2003, the *in vitro* an *vivo* antimalarial (Onabajo et al., 1983; Tona et al, 2001; Cimanga et al., 2008), antioxidative (Cimanga et al., 1999, 2021), cardioinhibitory (N'Guessan et al., 2002) and immunologic (Mankele et al., 2006), antibacterial (Mbamu et al., 2018), antidiarrheal (Mbamu et al., (2018) activities. The antiamoebic and spasmolytic activities of this medicinal plant were partly reported by (Cimanga et al., 2006a,b, 2018) since all isolated flavonoids and iridoids were not included and tested in this previous study. Isolated compounds concerned anthraquinones (Cimanga, 1997), flavonoids (Cimanga et al., 1995a, 1997) and iridoids (Cimanga et al., 2003).

Thus, the present study was undertaken to evaluate the spasmolytic activitY of other isolated chemical constituents form *M. morindoides* leaves including flavonoids and iridoids. The cytotoxic effect of all samples against Vero cells is also described

2. MATERIALS AND METHODS

2.1. Plant material

Fresh leaves of *Morinda morindoides* (Baker) Milne-Redh. (Rubiaceae) were collected in Kinshasa, Democratic Republic of Congo (DR-Congo) and the plant was identified for the first time in the Institut d'Etudes et de Recherches en Agronomie (INERA) of the University of Kinshasa in October 1990. A voucher specimen (MN 04122004MMSL) of the plant has been deposited in the herbarium of this institute. For the present study, a new batch of plant material was collected in the same place in April 2017. Fresh leaves were used in this study since this state of plant material is that used by traditional healers to prepare their remedies according to their daily practices.

Figure 1: *Morinda morindoides* (Baker) Milne-Redh. (Rubiaceae) leaves, imature fruits, flowers and mature fruits.

2.2. Reagents

Methanol (purity 99.98%), ethyl acetate (purity 99.96%) and *n*-butanol (purity 99% extra pure) were purchased from Across Organic (USA), chloroform (purity 99.99%) was obtained from Fisher Scientific (UK). All solvents were with HPLC grade. Distilled water was used.

2.3. Preparation of extracts, fractions, isolation of flavonoids and iridoids.

20 g of fresh leaves were mixed with 150 ml distilled water and boiled for 15 min at 100°C on a hotplate. The mixture was cooled and filtered. The filtrate was evaporated in *vacuum* to give dried extract denoted as extract AE (12.32g, 61.60%). An amount of extract AE (10 g) was dissolved in 100 ml distilled water, filtered on paper filter Whatman N° 1 and successively and exhaustively extracted with chloroform, ethylacetate and *n*-butanol. Each fraction was evaporated in *vacuum* yielding corresponding dried residues denoted as extracts AE-1 (1.58, 15.80%), AE-2 (2.16g, 21.60%) and AE-3 (2.46g, 24..60%) respectively. The residual aqueous phase was also treated as described above yielding a dried residue denoted as extract AE-4 (2.76g, 27.6%). Flavonoids and iridoids were isolated from 80% methanol extract (500 mg of plant material) using different chromatographic technics and identified by different conventional spectroscopic methods as previously described by (Cimanga, 1997). The identified flavonoids were quercetin (15.6 mg), Quercetin 7,4-demethylether (5.4 mg), luteolin 7-O-glucoside (6.3 mg), apigenin 7-O-glucoside (25.2 mg), quercetin 3-O-rhamnoside (10.5 mg), kaempferol 3-O-rhamnoside (14.1 mg), quercetin 3-O-rutinoside (34.5 mg), kaempferol 3-O-rutinoside 915.2 mg), chrysoeriol (22.8 mg) and chrysoeriol-7-O-neohesperidoside (84 mg). Iridoids were isolated by HPLC Gilson equipped with a RP10 column using MeOH/H_2O as mobile phase resulting in the obtaining iridoids identified as gaertneric acid (11 mg), gaertneroside(15 mg), aceylgaertneroside (43 mg), dehydrogaretneroside (5 mg),

dehydromethoxygaertneroside (9 mg), epoxygarteneroside (24 mg), epoxymethoxygaertneroside (5 mg) and methoxygaertneroside (12 mg) identified by different spectroscopic methods. These compounds were white with a bitter state (Cimanga, 2003).

2.3. Spasmolytic testing

Guinea-pigs were anesthetized and sacrificed by cervical displacement followed by exsanguination. The ileum was dissected out (2-3 cm long), plentifully washed with distilled water and suspended in an organ bath (50 ml) containing Tyrode's solution (mM: KCl:2.2, $MgCl_2$:0.11, $NaH_2PO_4.2H_2O$:0.42, $CaCl_2$:1.8, NaCl:137, $NaHCO_3$:11, glucose:5.6) or depolarizing solution rich in KCl (DSR-KCl) (mM: NaCl:2.7, KCl:100, $NaHCO_3$:15, $CaCl_2$:1.25, $MgCl_2$:12.5, glucose:11) gassed with 95% O_2 and 5% CO_2.[23,46]

The isolated tissue was allowed to equilibrate for 30 minutes under a resting tension of 0.5 g in Tyrode's solution or in DSR-KCl before exposure to drugs and tested samples. To evaluate spasmolytic activity, the tissue was first exposed to 5.10^{-7} M acetylcholine (ACh) or DSR-KCl to have tree equivalent contractions and the tissue was plentifully washed with Tyrode's solution to eliminate the presence of agonists in organ bath. To evaluate the spamolytic activity, 2 mg of each sample were dissolved in 2 ml distilled water to have respective stock solution of 1 mg/ml. After 3 ml of physiological solution was removed in organ batch and replaced by 2 ml of tested sample in organ bath (40 µg/ml in organ bath) and left in contact with isolated guinea-pig ileum for 15 minutes. After, 1 ml of agonist was then added in batch to induced contractions of isolated organ.

The effects of chloroform fraction and constituents (flavonoids and iridoids) on the responses elicited by both agonists were recorded. The responses were recorded via a frontal writing lever on kymograph paper (Scientific and Research Instruments Ltd. England). The experiment was repeated tree times and mean percentage inhibition of both agonist contractions in the presence of each tested sample was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Cag- Cts}}{\text{Cag}} \times 100$$

Where Cag is the amplitude level of contractions induced by agonist in cm and Cts is the amplitude level of contractions induced by tested sample (Cimanga et al., 2010; Ali et al., 2014; Silva et al., 2017). Effective doses (ED_{50}) values were derived using linear courbes responses-doses curves.

2.5.4. Cytotoxic evaluation against Vero cells

Cytotoxicity values of all

Compounds were determined by culturing Vero cells for 96 h in the presence of increasing amounts of each substance. Three wells for each concentration of each compound were used. Viable cells were determined by the trypan blue exclusion test. Results were plotted as a dose response curve and 50% cell growth inhibitory concentration (CC_{50}) was calculated (Cimanga, 1997a).

2.6. Statistical analysis

Results are presented as mean ± standard error of mean (S.E.M). Statistical analysis was carried out using one way analysis variance (ANOVA) followed by Turkey's multiple comparison tests where p value ≤ 0.05 was considered as statistically significant using Graph Pad Parism version 5.03 software.

3. RESULTS AND DISCUSSION

The historical antecedents date from the year 1504 when South American natives inhabiting the basins of the high Amazon and the Orinoco prepared a mixture of alkaloids termed curare. This substance was placed in the tips of arrows in order to hunt (prey paralyzing) and fight in wars. Curare produced muscle weakness, paralysis, respiratory failure, and at last death (Copy page, 2003). In 1800, Alexander von Humboldt, had shown that curare was made from the extracts of the species *Chondrodendron tomentosum* and *Strychnos toxifera*.

Figure 2: *Chondrodendron tometosum* (leaves ana stem) and *Strychnos toxifera* (leaves and fruits).

In 1935, the French physiologist Claude Bernard isolated the alkaloid d-Tubocurarine from the curare (Wintersteiner and Dutcher, 1943, Martínez-Pérez et al., 2018); and one year later, it was demonstrated that this

compound had the ability or capacity to inhibit acetylcholine contractions, blocking the transmission of nerve impulses to the muscles (Dale et al., 1936; Martínez-Pérez et al, 2018).

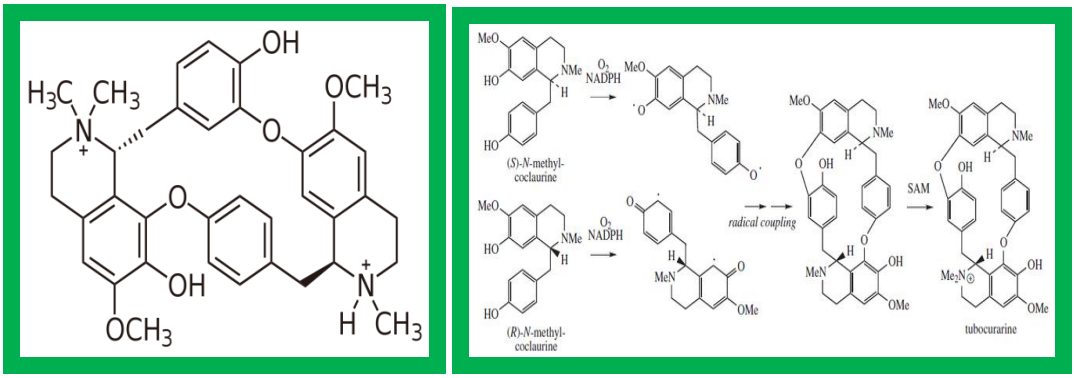


Figure 3: d-Tubocurarine (7',12'-dihydroxy-6,6'-dimethoxy-2,2',2'-trimethyltubocuraranium) and its biosynthesis.

In 1822, the pharmacist Rudolph Brandes isolated an impure alkaloid from *Atropa belladonna* (Solanaceae), which after purification was named atropine. Interestingly, it was not produced as a natural compound from the plant and was considered as a derivative generated from the alkaloid hyoscyamine during the purification process (Geiger and Hesse, 1833). Interestingly, Atropine had been obtained in small quantities in other members of the Solanaceae family such as *Datura stramonium*, *Duboisia myoporoides*, and *Scopolia japonica* and was at moment, considered as natural compound (Coulson and Griffin, 1967, 1968; Martínez-Pérez et al., 2018).

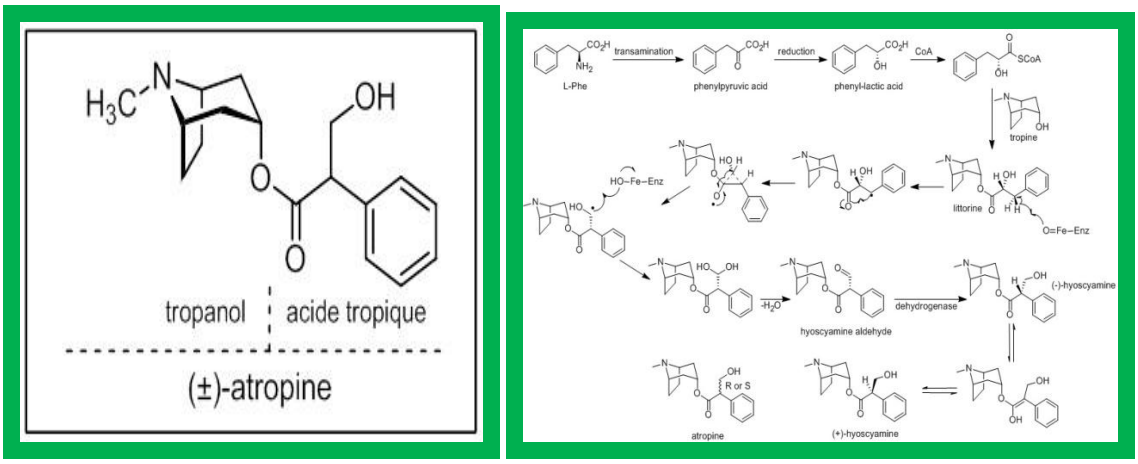


Figure 4: Atropine and its biosynthesis.

The use of the plant *Papaver somniferum* (opium poppy) (Papaveraceae) dated back to about 4000 BC. The word "opium" is of Greek (Unani) origin which was derived from opos (juice) and opion (poppy juice). Opium mostly postulated to came into Greece from Asia Minor and the ancient Greeks associated various divinities with opium, including Hypnos (sleep), Morpheus (dreams), Nyx (night) and Thanatos (the twin brother of Hypnos) (death). According to Unani literature, it was used alone or in combination with other medicines as compound formulation like Itrifal Muqawwi Dimagh, Sharbat khashkhash, and khamire khashkhash. etc (Masihuddin et al., 2018).

It was used as analgesic, narcotic, sedative, treatment of alcoholism, stimulant as well as nutritive, etc. Derivatives of opium alkaloids continued to play a major role as antitussives, antidiarrheals and analgesics. Opium was frequently mentioned in classical Unani literature for it pain relieving and sleep inducing action (Masihuddin et al., 2018).

Nowadays, the plant was only used to extract other alkaloids, such as noscapine and codeine used as antitussive, both discovered by the French pharmacist Pierre-Jean Robiquet in 1831 and 1832, respectively (Wisniak, 2013). Since the isolation of Morphine by Sertümer in 1803, some 40 alkaloids, representing different structural types of isoquinolines, had been isolated from opium. Morphine had analgesic effects. These alkaloids were combined with a number of acids including fumaric, lactic and the rare meconic acid. In addition, opium also contained a complex mixture of

substances including protein, sugar, fat, resin, rubber, wax and mucilaginous substances (Lindner, 1985).

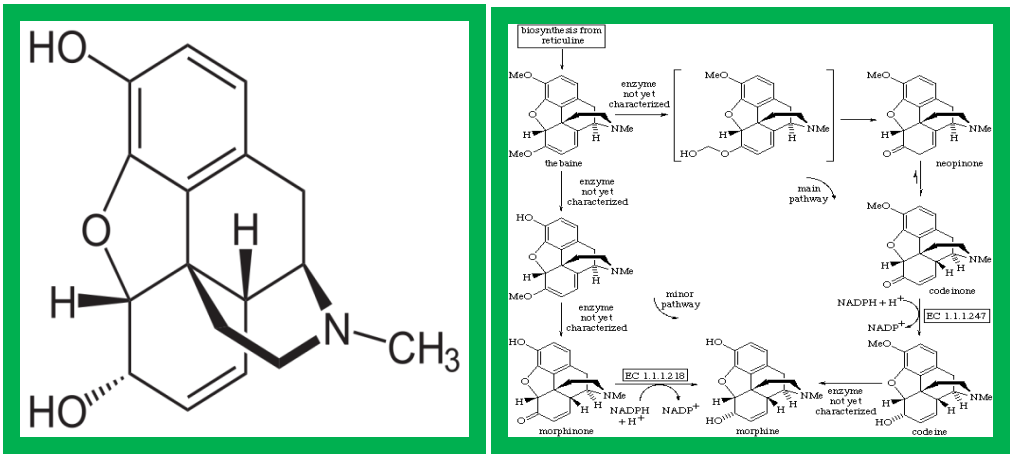


Figure 5: Morphine and its biosynthesis.

EC 1.1.1.218: morphine 6-dehydrogenase, EC 1.1.1.247: codeinone reductase (NADPH)

In 1848, Papaverine was another alkaloidic substance extracted from the same plant by the German chemist Georg Merck (Hayes, 2009; Martínez-Pérez, 2018) which was rarely used today because of the high doses needed (approximately 6 to 12 mg). It relaxed involuntary smooth muscle and increased cerebral blood flow. However, it was still used as a positive control in experimental pharmacological models with the purpose of studying antispasmodic activity of plant extracts and fractions and other.

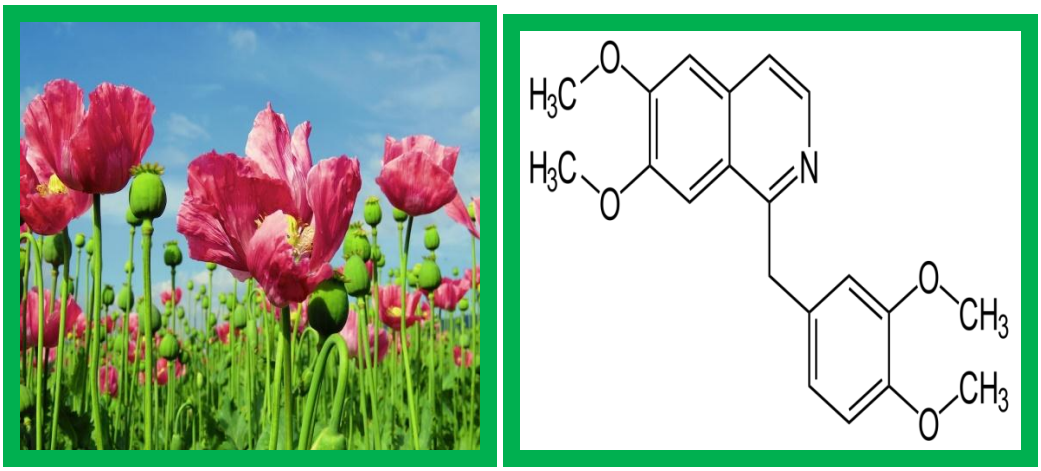


Figure 5: *Papaver somniferum*: flowers and opium and Papaverine.

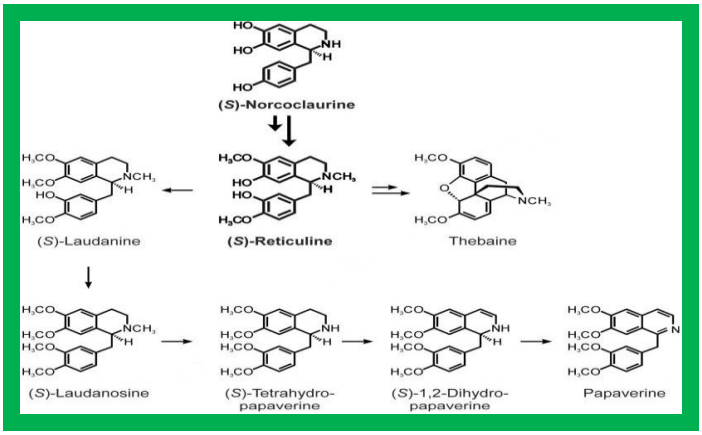


Figure Biosynthesis of papaverine.

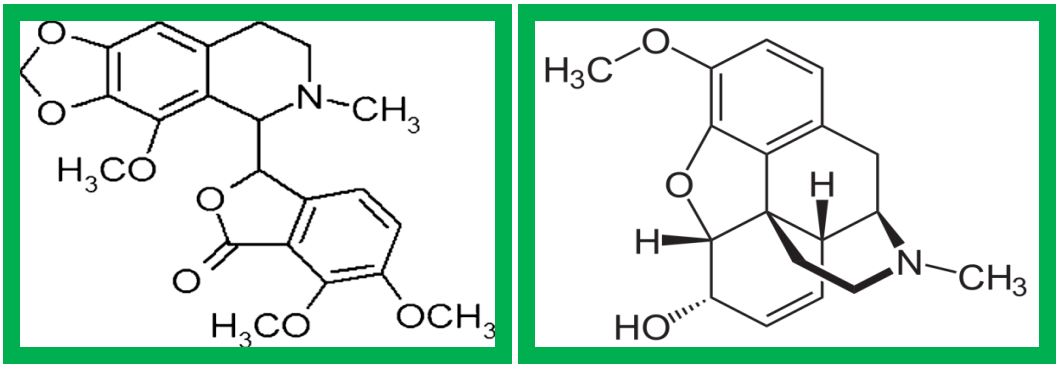


Figure 6: Noscapine and Codeine.

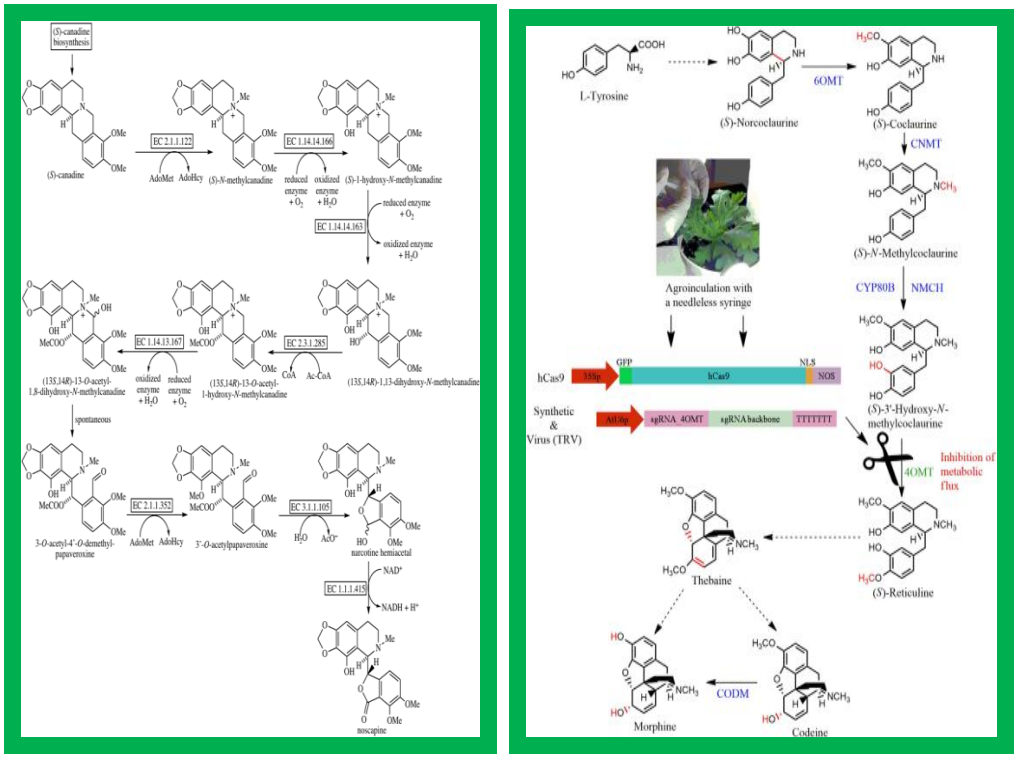


Figure 7: Biosynthesis of Noscapine, Codeine.

In the century, extracts and powders derived from *A. belladonna* were widely used as antispasmodics, but from the 1950s, these preparations were displaced by synthetic and semisynthetic anticholinergic compounds in order to obtain a better response (Grynkiewicz and Gadzikowska, 2008) such as Methocarbamol and Guaifenesin. On the other hand, a series of compounds such as Dantrolene, Glutethimide, Methaqualone, Chlormezanone, Metiprilone, and Ethchlorvynol were introduced to replace Meprobamate, which had been withdrawn from the market in 1960 due to problems resulting from use such as abstinence, addictions and overdoses.

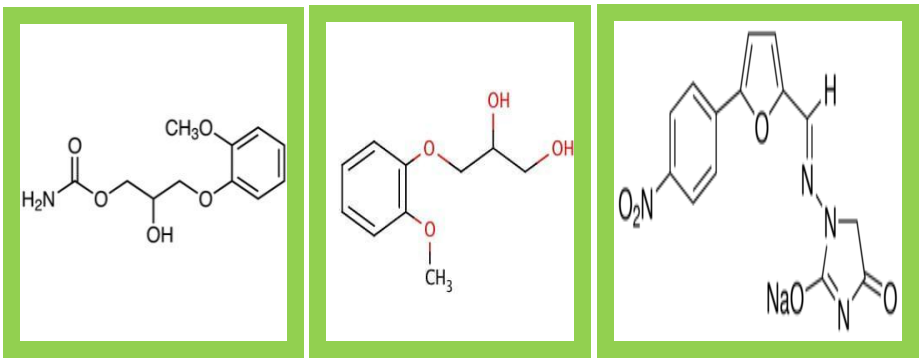


Figure 8: Methocarbamol, Guaifenesin and Dantrolene.

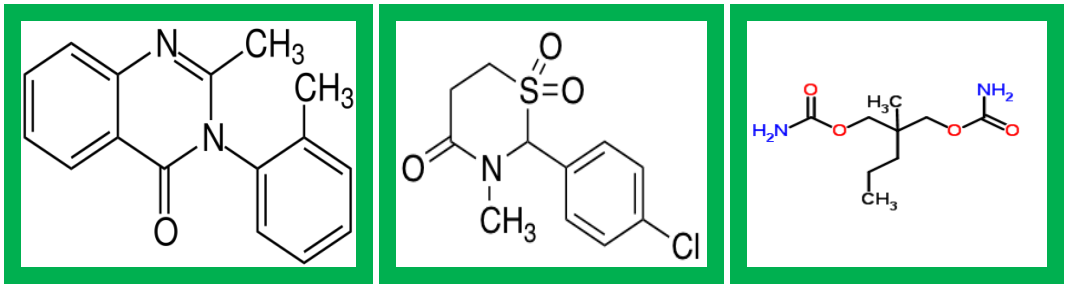


Figure 9: Methaqualone, Chlormezanone and Meprobamate.

In 1962, the Swiss chemist Heinrich Keberle synthesized baclofen, which can be obtained by reacting Glutarimide with an alkaline solution (Keberle et al., 1972). Glutarimide can also be found in plants such as *Croton cuneatus* and *C. membranaceus* (Euphorbiaceae) (Aboagye et al., 2000; Suárez et al., 2004; Martínez-Pérez et al., 2018).

Figure 10: Glutarimide, *Croton cuneatus* leaves and flowers), *C. membraceus* (leaves).

The arrival of the quaternary compounds of nitrogen group reinforced their peripheral anticholinergic activity offering also the advantages of being poorly absorbed in the gastrointestinal tract, producing a more powerful and longer lasting sedative effects unlike atropine (Warburton, 1969). For example, Ipratropium bromide was developed by the German company Boehringer Ingelheim in 1976 and used to treat asthma. This compound was obtained by reacting atropine with isopropyl bromide (Oates et al., 1988). Another quaternary compound was the *n*-Butylhyoscine bromide, which was possible to obtain by the organic synthesis of scopolamine and the Cimetropium bromide found in the *A. belladonna*.

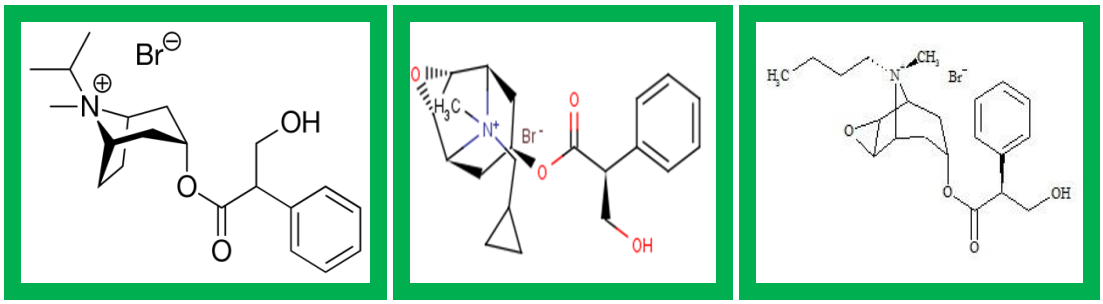


Figure 11: Ipratopium bromide, Cimetropium bromide and n-Butylhyocine bromide.

(Modignani et al., 1977; Martínez-Pérez et al., 2018). Although nowadays, the preparations of plant mixtures were no longer used for therapeutic purposes, these compounds formed.

Figure 12: *Atropa belladonna*: leaves, flowers and fruits.

a part of and served as the basis for modern pharmacology for their applicability as antispasmodics, analgesics and anesthetics (Martínez-Pérez et al., 2018).

Spasms were involuntary contractions of the muscles, which were normally accompanied by pains and interfered with the free and effective muscular voluntary activity. Muscle spasm can originate from multiple medical conditions and was often associated with spinal injury, multiple sclerosis, and stroke (Martínez-Pérez et al., 2018).

Spasticity and rigidity were caused by a disinhibition of spinal motor mechanisms. There were several scenarios where a muscle can produce a spasm: **(1)** unstable depolarization of motor axons, **(2)** muscular contractions persist even if the innervation of muscle was normal and despite attempts of relaxation (myotonia); **(3)** after one or a series of contractions, the muscle can decontract slowly, as occurring in hypothyroidism; and **(4)** muscles lack the energy to relax (Martínez-Pérez et al., 2018).

3.1. Spasmolytic activity of samples from *M. morindoides* leaves

In previous study conducted by Cimanga et al. (2010) on the spasmolytic activity of *M. morindoides* leaves, this activity for aqueous extract and its soluble fractions petroleum ether, diethylether, ethylacetate, *n*-butanol and residual aqueous, as well as crude flavonoids and saponins was reported. Three isolated flavonoids including quercetin, quercetrin and rutin, and 2 iridoids epoxygaertneroside and gaertneroside were also tested.

Results from this study indicated that aqueous extract and its petroleum ether potentialised the effects of both agonists acetylcholine (ACh) and depolarizing solution rich in KCl (DSR-KCl)(% > 100%). Diethylether soluble fraction rich in steroids and terpenes completely inhibited contractions of isolated guinea-pig ileum induced by ACh (% inhibition: 100) and showed low inhibitory effect against contractions induced by DSR-KCl (% inhibition: 24). Ethylacetate soluble fraction rich in flavonoids exhibited good spasmolytic activity with a percentage inhibition > 70% against both agonists while *n*-butanol fraction rich in saponins exhibited moderate activity (50 < % inhibition < 20% against both agonists). This showed smooth muscle relaxant (antispasmodic) activity may be mediated through calcium antagonistic effect as high K^+ (>30 mM) was known to cause smooth muscle contractions through opening of voltage-dependent L-type Ca^{++} channels, thus allowing influx of extracellular Ca^{++} causing a contractile effect as also reported by Ali et al., (2014).

The three flavonoids and two tested iridoids cited above were previously reported to exhibit spasmolytic activity by producing more than 72% and 67% inhibition respectively, of contractions induced by both agonists on isolated guinea-pig ileum. This summary indicated that *M. morindoides* leaves contained spasmolytic principles, but all isolated flavonoids and iridoids were not included and tested for this biological activity and this was now completed in the present study.

Results for the present study were presented in Table 2. In order to verify the presence of cholinergic components in chloroform fraction, it was monitored its effect on ACh-induced contractions of isolated guinea-pig ileum. Then, the chloroform soluble fraction rich in steroids and terpenoids which was not tested before and was found in the present study able to inhibit contractions induced by ACh and DRS-KCl on isolated guinea-pig ileum by 97.35±0.02 and 85.30±0.05% with effective doses values (ED_{50}) of 10.27±0.03 and 11.72±0.06 µg/ml respectively. It caused thus, significant relaxation of the ileum stimulated by ACh and high concentrations of KCl (DSR-KCl).

For flavonoids, results revealed that chrysoeriol produced more than 70% inhibition of contractions induced by both agonists while kaempferol and luteolin showed 70.32±0.03 and 73.25 ±1.12% inhibition respectively of contractions induced only by ACh. Against this agonist, the remaining tested flavonoids mainly glycoside derivatives, inhibited its contractions induced on the isolated organ by producing 60 % < % inhibition < 70%. The high percentages were showed by chrysoeriol-neohesperidoside, luteolin-7-O-glucoside and kaempferol-7-O-rutinoside (70 < % inhibition < 66) compared the remaining other flavonoids (Table 1).

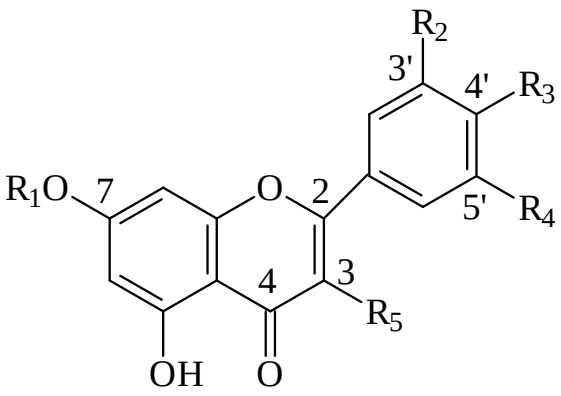
Table 2: Spasmolytic activity of samples from *M. morindoides* leaves.

Samples	% I ACh	ED_{50},µg/ml	%I DSR-KCl	ED_{50},µg/ml
$CHCl_3$	97.35±0.02	10.27±0.03	85.30±0.05	11.72±0.02
Flavonoids				
Apigenin	67.85±0.02	31.00±0.04	65.18±0.08	30.75±0.03
A-3-O-glucoside	60.25±0.05	33.35±0.06	58.96±0.04	34.00±0.04
Chryoeriol	77.58±0.03	26.03±0.07	74.68±0.02	27.03±0.01
C-7-O-neohesperidoside	65.24±0.07	30.75±0.02	62.57±0.03	32.00±0.05
Luteolin	73.25±1.12	27.45±0.03	69.35±0.05	29.00±0.02
L-7-O-glucoside	68.54±0.05	29.25±0.07	66.57±0.08	30.06±0.04
Kaempferol	70.32±0.03	28.54±0.02	67.52±0.04	29.78±0.02
K-3-O-Rhamnoside	63.54±1.50	31.54±0.03	60.21±1.14	33.62±0.01
K-3-O-Rutinoside	68.54±0.04	29.36±0.05	65.31±0.02	30.66±0.04
Morindaoside	64.12±1.24	32.21±0.04	61.24±0.07	32.71±0.03
Iridoids				
Acetylgaertneroside	78.17±0.02	25.63±0.04	75.92±0.05	26.40±0.04
aertneric acid	72.25±0.02	27.63±0.02	70.25±0.03	28.42±0.04
Gartneroside	70.83±0.04	28.68±0.00	67.66±0.01	29.75±0.01
Methoxygaertneroside	76.30±0.04	26.15±0.03	74.89±0.02	26.66±0.03
Epoxygaertneroside	72.25±0.03	27.56±0.02	70.70±0.01	28.02±0.00
Epoxymethoxygaertneroside	74.25±0.02	26.87±0.01	72.69±0.01	27.58±0.05
Dehydrogaertneroside	78.36±0.02	25.57±0.04	75.47±0.00	26.64±0.02
Dehydromethoxygaertneroside	71.25±0.05	28.15±0.03	68.67±0.03	29.22±0.00
Atropine sulfate	100.00±0.00	3.75±0.02	-	-
Papaverine chlorhydrate	100.00±0.00	4.15±0.04	98.96±0.00	4.35±0.02

% IACH: % inhibition acetylcholine, ED_{50}; effective dose 50, % IDSR-KCl: % inhibition of depolarizing solution rich in KCl, A: apigenin, C: chrysoeriol, K: kaempferol, L: luteolin. $CHCl_3$ soluble fraction from the partition of aqueous extract.

Against DSR-KCl inducing contractions on isolated guinea-pig ileum, except chrysoeriol which showed % inhibition between 70 and 80% inhibition of contractions provoked by this agonist, results indicated that the remaining tested flavonoids including aglycones and glycosides were able to inhibit its effect by producing percentage inhibition ranging from 60 to 70%. The high activity was produced by luteolin and its -7-O-glucoside, kaempferol and its -3-O-rutinoside producing showing more than 65% inhibition against this agonist. The lowest activity was shown by apigenin-3-O-glucoside (58.96±0.04 % inhibition).

In general, flavonoid aglycones showed high spasmolytic activity against both agonists than their corresponding glycoside derivatives. Our results were in good agreement with Gharzouli et al. (2004) concerning the spasmolytic activity of apigenin. Moreover, these flavonoids exhibited spasmolytic activity with ED_{50} values ranging from 26.03 to 33.35µg/ml and 27.03 to 34.00 µg/ml against ACh and DSR-KCl agonists respectively (Table 2).



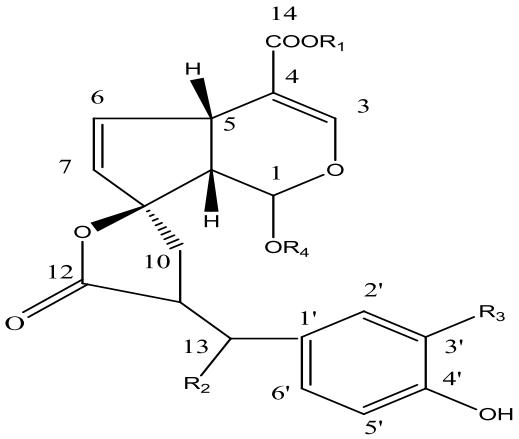
	R_1	R_2	R_3	R_4	R_5
1. Apigenin	H	H	OH	H	H
2. A-3-O-glucoside	Glc	H	OH	H	H
3.Chrysoeriol	H	H	OH	OCH_3	H
4. C-7-O-neohesperidoside	Rha-(1→2)-Glc	H	OH	OCH_3	H
5. Kaempferol	H	H	OH	H	OH
6. K-3-O-rhamnoside	H	H	OH	H	Rha
7.K-3-O-Rutinoside	H	H	OH	H	Rha-(1→6)-Glc
8. K-7-O-neohesperidoside	Rha-(1→2)-Glc	H	OH	H	OH
9. Quercetin	H	OH	OH	H	OH
10. Q-3-O-rhamnoside	H	OH	OH	OH	O-Rha
11. Q-3-O-rutinoside	H	H	OH	OH	Rha-(1→6)-Glc
12.Q-7,4' dimethyl-ether	OCH_3	H	H	OCH_3	OH

A: apigenin, C: chrysoeriol, K: kaempferol, Q: quercetin

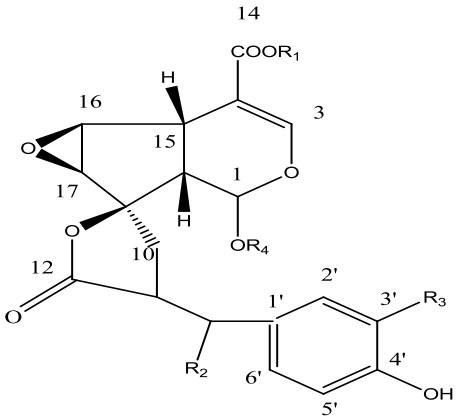
Figure 13: Structures of spasmolytic flavonoids isolated from *M. morindoides* leaves.

For iridoids tested in the present study, it was observed that all tested compounds exhibited high spasmolytic activity by producing more than 72% inhibition of contractions induced by both agonists on isolated guinea-pig ileum. The high activity was produced by acetylgaertneroside (78.17±0.02 and 75.92±0.05% inhibition respectively), dehydrogaertneroside (78.36±0.12 and 75.47±0.24% inhibition respectively) and their activity seemed to be comparable and did not significant difference ($p > 0.05$). Methoxygaertneroside (76.30±1.04 and 74.89±0.02 % inhibition respectively), epoxygaertneroside (72.25±0.04 and 70.70±001 % respectively) and gaertneric acid (72.25±1.23 and 70.25±0.03 % inhibition), gaertneroside (70.83±0.04 and 67.66±0.01) also exhibited interesting spasmolytic activity taking account of their percentage inhibition level.

Gartneroside and epoxygaertneroside were inserted in the present study for a good discussion on structure spasmolytic activity relationship. Thus, a structure spasmolytic activity-relationship for iridoids showed that the presence of acetyl group in position C-14 increased the activity (acetygaertneroside compared to gaertneroside and gaertneric acid, the presence of H group in C-14 decreased the activity (gaertneric acid compared to actylgaertneroside), the presence epoxy group between C-6-C-7 position was also favourable to the increase of activity (expoxymethoxygaertneroside compared to methoxygaertneroxide), the presence of methoxy group in C-3' position contributed to the increase of activity (epoxymethoxygaertneroside compared to epoxygaertneroside), the absence of methoxy in C-3' position was favourable to the increase of activity (dehydrogaertneroside compared to dehydrmetoxygaertnerodise).



R_1	R_2	R_3	R_4		
1	CH_3	OH	H	Glc	: Gaertneroside
2	CH_3	OH	H	6-acetyl-Glc	: Acetylgaertneroside
3	CH_3	=O	H	Glc	: Dehydrogaertneroside
4	CH_3	=O	OCH_3	Glc	: Dehydromethoxygaertneroisde
5	H	OH	H	Glc	: Gaertneric acid
6	CH_3	OH	OCH_3	Glc	: Methoxygaertneroside



R_1	R_2	R_3	R_4		
7	CH_3	OH	H	Glc	: Epoxygaertneroside
8	CH_3	OH	OCH_3	Glc	: Epoxymethoxygaertneroside

Figure 14: Structures of spasmolytic iridoids isolated from *M. morindoides* leaves.

The presence of =O group in position C-13 and methoxy group in C-3' decreased the activity (dehydromethygaertneroside compared to dehydrogaertneroside or the presence =O group in C-13 and H group in C-3' increased the activity (dehydrogaertneroside compared dehydromethoxygaertneroside). In addition, these iridoid compounds displayed spasmolytic activity with ED_{50} values between 25.13 to 28.15 µg/ml and 26.40 to 29.22 µg/ml against ACh and DSR-KCl respectively (Table 2).

Figure 15 showed the oontractile responses to ACh and DSR-KCl increasing concentrations (5-30 µg/ml) on isolated guinea-pig ileum preparations. The tissues were prepared with ACh and K^+ free Tyrodes solution in the absence or in the presence of $CHCl_3$ fraction . Data are expressed as the % of the maximal contractile response to both agonists indicated statistically significant difference with the ANOVA of repeated measures and the *post-hoc* Stdudent's t test ($p \leq 0.05$). Considering that 5-HT in the intestine caused contractions and that smooth muscle contraction was calcium dependent, it was possible that $CHCl_3$ fraction from *M. morindoides* leaves contained a calcium antagonist or an inhibitor of the 5-HT receptors. In addition to this, considering that this selected fraction was effective in diminishing isolated ileum contractions, further studies may aim at determining the compounds involved in the spasmolytic activity of the fraction. It can be claimed that sewlected chloroform fractiom was capable of mediating spasmolytic effects on isolated guinea-pig ileum through the inhibition of a wide range of contractile stimuli, such as neurotransmitters (ACh and DSR-KCl).

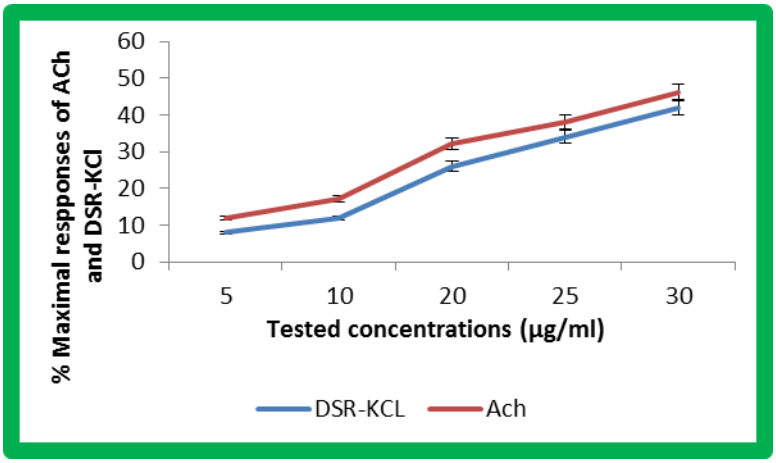


Figure 15: Maximal responses to ACh and DSR-KCl contractions induced by $CHCl_3$ fraction from *M. morindoides* leaves.

This suggested that the fraction relaxant effect on the ileum was not caused by a specific receptor, but rather by either a general receptor inactivation or a depolarization of the membrane. Our results were in good agreement with Garín-Aguilar et al., (2014) who reported the same effect with extracts from *Alternanthera repens* aerial parts.

To assess whether the spasmolytic activity of aqueous extract, flavonoids and iridoids were also mediated through Ca^{2+} channel blockade, high concentration of K^+

(100 mM) was used to depolarize the preparation (Gilani et ql., 2006a). Moreover, the use of high K^+ concentration higher than 30 mM, was known to produce smooth muscle contractions through the opening of voltage-dependent L-type Ca^{2+} channels, thus allowing influx of extra-cellular Ca^{2+} producing contractile action (Gilani et al., 2006). This activity consisted of the depolarization of the ileum preparations and the production of myo-contractions by opening the voltage-dependent Ca^{2+} channels, thus allowing the influx of extracellular Ca^{2+} and causing a contractile effect (Garín-Aguilar et al., 2014). Thus, any substance which caused inhibition of the high K^+ induced contractions was considered as an inhibitor of the Ca^{2+} influx or a Ca^{2+} influx blocker as explained by (Godfraind et al., 1998). Calcium antagonists were considered as an important therapeutic group and were characterized by their dose-dependent inhibition of slow entry of this cation (Fleckenstein, 1977). It can be considered that the spasmolytic effect of *M. morindoides* isolated constituents, as evident by the relaxation of high K^+-induced concentrations, may be due to the channel blockade as also reported for the fractions and extracts of *Euphorbia granualta*, and mediated possibly through Ca^{2+} antagonist effects which can explain their therapeutic usefulness in hyperactive gut disorders, such as abdominal coli and diarrhoea (Ahmad et al., 2012). *M. morindoides* $CHCl_3$ fraction and isolated constituents tested in the present study caused the relaxation of high K^+ in a dose-dependent manner, indicated the involvement of Ca^{2+} channel blocking activity in spasmolytic activity because substance inhibited high K^+ induced contractions was denoted CCB (calcium channel blocker) (Ali et al., 20114; Asifa et al., 2017). Moreover, in KCl-induced contractions, the voltage dependent calcium channels were involved and the existence of L-type voltage dependent calcium channels in pig ileum had been reported (Naseri et al, 2008).

Acetylcholine (ACh) was a neurotransmitter released by the parasympathetic nervous system. Its action in the gastrointestinal tract (GIT) involved the stimulation of nicotinic and muscarinic acetylcholine receptors. The M2 and M3 receptor subtypes played an essential physiological role in the smooth muscle contraction/relaxation of the GIT. The following mechanisms helped to explain the increased GIT contractility induced by synthetic drugs or medicinal plant extracts: **(1)** stimulation of the ACh release from the cholinergic nerve endings, **(2)** stimulation/inhibition of the acetyl cholinesterase enzyme (AChE) at the neuro-effector junction, and **(3)** direct activation/inactivation of the muscarinic receptors of the smooth muscles, including those of the GIT (Garín-Aguilar et al., 2014).

Morinda morindoides extracts, fractions, flavonoids and iridoids caused concentration-dependent inhibition of the spontaneous and high K^+ induced contractions, with EC_{50} values from 11.72±0.02 to 35.72±0.02 µg/ml. In similar pattern, Papaverine, a standard Ca^{2+} antagonist (Ali et al., 2013), relaxed the spontaneous and high K^+ induced contractions, with EC_{50} value of 4.35±0.02 µg/ml. This showed smooth muscle relaxant (antispasmodic) activity may be mediated through calcium antagonistic effect as high K^+ (>30 mM) was known to cause smooth muscle contractions through opening of voltage-dependent L-type Ca^{2+} channels, thus allowing influx of extracellular Ca^{2+} causing a contractile effect (Ali et al., 2014) and the substance which caused inhibition of high K^+-induced contraction was considered to be an inhibitor of Ca^{++} influx (Ali et al., 2014). The spasmolytic effect can interfere with the contraction mechanism of extracellular Ca^{2+} internalization or release from Ca^{2+} depots in the sarcoplasmic reticulum (Garín-Aguila et al., 2014).

Periodic depolarization and repolarization of the tissues due to the influx of calcium into sarcoplasmic reticulum trough voltage-dependent calcium channel were known as events responsible for spontaneous intestinal responses and suggested that substances that inhibited KCl-induced contractions of isolated organ acted via blocking the channels (Vadivel et al. 2017).

In addition, the spasmolytic effects of these constituents isolated from *M. morindoides* were completely reversible after plentiful washing of isolated guinea-pig ileum with Thyrode's solution and restimulation separately with each agonist suggesting that their effects were possibly not accompanied with binding to Ca^{2+} channels or/and entering to smooth muscle cells. The reverse results were also reported by Naseri et al. (2008) on the spasmolytic activity of Onion (*Allium cepa* L.) peel extract on rat ileum. Existence of anticholinergic activity in these *M. morindoides* samples provided also the sound justification of their antidiarrheal properties.

Different secondary metabolites including flavonoids (Ogongbamila et al., 1990; Capasso et al., 1991; Galvez et al., 1993a ; Morales el 1994; Coldzada et al., 1999), iridoids (, Trute et al., 1997; Urtie et al., 1999; Fleer et al., 2007; Tundi et al., 2008), alkaloids (de Meiros et al., 1991, Capasso et al., 1997; Martin et al, 1993), coumarins (e-Shafae et al., 1988) [63], saponins (Corea et al., 2005, Naz et al.., 2016), steroids and terpenoids (Assifa et al., 2017; Marinez-Pérez et al., 2018) and tannins such as proanthocyanidins (Galvez et al., 1993b) were previously reported to exhibit spasmolytic activity in different experimental models.

Papaverine chlorhydrate tested at 40 µg/ml in organ bath produced 100% inhibition of ACh and DSR-KCl while atropine sulphate produced only 100% inhibition against ACh and was devoid with effect against DRS KCl-induced contractions of isolated guinea-pig ileum. Thus, all tested samples form *M. morindoides* leaves had papaverine-like effect.

When compared with standard spasmolytic agents atropine and papaverine, it was found that all isolated

constituents from *M. morindoides* leaves had comparatively less potent spasmolytic effect than standard drugs. As many spasmolytic drugs available in market had side effects such as urinary retention, tachycardia, urinary hesitancy, mydriasis, hypersensitivity effects and blurred vision (Goodfraind et al., 1998; Barakat et al., 2013), *M. morindoides* constituents being origin natural drugs with high degree of safety, tolerability and efficacy, could be considered as suitable alternative to existing drugs, as well as could be considered as new members of spasmolytic family.

Spasmolytic compounds exerted their activity in different ways, such as through inhibition of the response to the neurotransmitters 5-hydroxytryptamine (5-HT) or serotonin and acetylcholine. However, the spasmolytic effect of these compounds was also attributed to capsaicin-sensitive neurons, the participation of vanilloid receptors, the activation of K^+ ATP channels, the blockade of Na^+ channels and muscarinic receptors, the reduction of extracellular Ca^{2+}, and the blockade of Ca^{2+} channels (Gilani et al., 2009; Mehmod et al, 2001, Chattida et al., 2018). The above was merely a reflection of the ambiguity of the studies showing the mechanisms of action of the spasmolytic compounds (Taqvi et al., 2009). For example, the hydroalcoholic extract of *Marrubium vulgare* showed spasmolytic effect, having the ability to inhibit the neurotransmitters acetylcholine, bradykinin, prostaglandin E2, histamine, and oxytocin, whereas a dual effect of antidiarrheal and laxative activities was reported in *Fumaria parviflora* (Sclnifer et al., 1996).

4. CONCLUSION

The results from this study clearly demonstrated that isolated constituents including flavonoids and iridoids from *M. morindoides* leaves possessed spasmolytic activity with different magnitudes. The spasmolytic activity displayed by iridoids was higher compared to flavonoids and all constituents can act in synergistic manner. These isolated constituents can be considered as active principles responsible for the spasmolytic activity of this medicinal plant part used in traditional medicine to treat diarrhea and its use for this medical purpose seemed to be more support and justify as it had been previously reported for aqueous extract and other constituents from *M. morindoides* leaves (Cimanga et al., 2010).

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