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Anti Food Poisoning Pathogenic Bacteria in Correlation to Coumarins and Flavonoids of *Atropa belladonna* Field Plant and *in vitro* Cultures

Hanan Abd Al-Hay Al-Ashaal[a]*, Azza Abuelnaga[b] Ahmed A. El -Beih[a], Sahar Salah el din[a]

[a]*Drugs and Pharmaceutical Industries Research Division, National Research Centre, Giza, Egypt (Affiliation ID:60014618)*
[b]*Microbiology and Immunology Department, Veterinary Division, National Research Centre*

Abstract

Aim: Investigation of the antimicrobial activity of *Atropa belladonna* leaves and *in vitro* callus extracts against food borne bacteria.
Method: *A. belladonna* leaves were used for *in vitro* study on Murashige and Skoog media supplemented with growth regulators. Coumarins and flavonoids phenolics were extracted from mother field plants, callus and differentiated plants with 70% methanol after segregation of tropane alkaloids. The examined bacteria strains included *S. aureus*, *E. coli*, *S. typhi,* C. *jejuni*, and *L. monocytogenes*
Results: HPLC analyses illustrated the presence of bergapten, xanthotoxin and umbelliferone coumarins. The analyses also disclosed the presence of rutin, Quercetin 3-O-rhamnoside, kaempferol rhamnoside, and kaempferol flavonoids. Antimicrobial investigation showed that both mother plant and *in vitro* callus extracts showed potent anti microbial activity against the selected strains of food poisoning bacteria comparing to broad spectrum antibiotics.
Conclusion: Present outcomes highlight the prospect role of *in vitro* cultures to produce the coumarins and flavonoids in steady regular technique. Current findings also explore the effectiveness of these metabolites as natural anti food borne bacteria; the causative agents of many risky diseases. Present results could be start guide for producing plant derived antibacterial and help in solving the problem of resistance of bacteria to currently used drugs.

Key words: *Belladonna,* Callus, Coumarins, Flavonoids, Foodborne bacteria.

INTRODUCTION

Since early history until now, *Atropa belladonna* has been used in traditional medicine for its diverse biological activities. In ancient Egypt, the plant was used by healer to treat air way diseases as asthma and pulmonary aliments. Solanaceae extracts have been used from the fourth century before Christ and were familiar through Roman, Islamic and Egyptian states [1]. The plant is famous after its tropane alkaloids content that have very important multidiscipline pharmacological effects [2]. Phenolics are another important secondary metabolites present in the plant including flavonoids, tannins and coumarins. Plants obtained phenolics were reported to suppress cancer initiation and development *via* different mechanisms including repressing oncogenic proteins and promoting tumour suppressor proteins expression. In addition, phenolics struggle several oxidative stress associated diseases [3]. Antiviral potential of phenolics against diverse of viruses and cancer associated virus, is quite documented [4, 5]. Another essential property of phenolics is antimicrobial character. Phenolics are synthesized in the plants as natural antioxidant and antibacterial against bacterial attack [6]. Regarding *Atropa belladonna,* methanolic extract of aerial parts except for berries and flowering tops showed antibacterial effect versus gram-positive (*Staphylococcus aureus*) and gram negative (*Klebsiella pneumonia* and *Escherichia coli*) strains [7].Whereas, ethanolic root extract was more significant as antimicrobial against *S .aureus* than *E. coli* and had no antibacterial effect against *S. typhi* [8]. In the same line, it was reported that methanolic extract of aerial parts of the plant exhibited acaricidal effectiveness against *Rhipicephalus microplus* [9]. Many authors returned the antimicrobial activity to tropane alkaloids [9,10]. On the other hand, Food poisoning illnesses are commonly infectious or toxic in their nature and are caused by microorganisms as bacteria, viruses, parasites or by chemicals that get inside the body *via* contaminated food and water. Consequently, seeking for effective antibacterial compounds is essentially required. As *A. belladonna* is a potential source of worth active compounds and one of those plants bearing phenolics, *in vitro* cultures are being investigated as substitutional process for production of these active constituents. The majority of *in vitro* studies focused on the production of tropane alkaloids [11, 12]. In our previous study, *in vitro* production of tropane alkaloids and their pharmacological effects were investigated [2]. Therefore, the aim of the present work is continue studying the potential role of *A. belladonna cultures* as prospective source of coumarins and flavonoids phenolics. Besides, the examination of the antimicrobial efficiency of callus, and mother plant extracts.

MATERIAL AND METHODS

Plant material

Atropa belladonna L. leaves were derived from the Medicinal Plant Farm of the Faculty of Pharmacy, Cairo University. The plant was authenticated by Dr. Salwa Kawashty, Department of Phytochemistry and Plant Systematic, National Research Centre (NRC).

Chemicals, standards and apparatus

Coumarins (bergapten, xanthotoxin and umbelliferone) and flavonoids (rutin, Quercetin -O-rhamnoside, Kaempferol-O-rhamnoside, and Kaempferol), all were from Sigma and were kind gifts from Dr. Tahya Elkobesy and Dr. Mohamed El Elraiy, NRC. Erythromycin, amoxicillin, gentamycin and nalidixic acid antibiotics (Oxoid Co.). The antibiotics were kind donations from Dr. Nayera Ahmed, Natural and Microbial products Dep. and Dr Ayman Samy, Microbiology and Immunology Dep, NRC. Chemicals for *In vitro* study are tissue culture grade. Solvent for analysis are HPLC analytical grade.
HPlC: Thermo ultimate 3000 High Performance Liquid Chromatography with ultraviolet (UV) detector, ODS C18 Reporsil analytical column (5µm, 4.6×250 mm), GmbH, Germany.

In vitro plant tissue culture study

Establishment of callus and differentiation cultures was carried out according to the protocol adopted in our previous work [2]. The leaves were cleaned with tap water and sodium hypochlorite 10% for 20 min. then immersed in70% alcohol for seconds and finally rinsed with distilled water. For callus cultures, the sliced leaves were aseptically cultured in MS media contained different concentrations of Kin, BA, and IAA. For differentiation, leaves were cultured in Ms media with BA and IAA at serial concentrations.

Extraction and identification of coumarins and flavonoids

Calli, regenerated leaves, stems and roots in addition to intact mother leaves (50 mg each) were dried at a temperature not exceeding 40°C and then ground in coarse powder. The dried materials were extracted with chloroform: methanol: 25% ammonia (15: 15: 1 v/v/v) for tropane alkaloids extraction. The remaining materials after segregation of tropane alkaloids were then extracted with 70% methanol. Methanol extracts were evaporated under reduced pressure and temperature. The residues were kept for coumarins and flavonoids analyses.

HPLC analyses

Five milligrams of the residue of tested samples were dissolved in 80% methanol and filtered through 0.45 µ Millipore membrane filter then injected in HPLC column for qualitative and quantitative analyses.

For coumarins: The column temperature was set at 25 ◦C. A mobile phase isocratic (MeOH-H2O; 49:51, v/v) acidified with phosphoric acid 0.05% (v/v), flow rate of 1.0 ml/min. The (UV) detector was adjusted at 345 nm. Run time 15min.

For flavonoids: Column temp. 25◦C, analyses were carried out using linear gradient mobile phase; 100% acetonitrile and0.1% acidified water with formic acid (0-1 min), 100%-70% 0.1% acidified (1-30 min), 70% acetonitrile- 20% 0.1% acidified water (30-35 min). Flow rate was 0.3 mL/min and UV detector was set at 254 nm. Run time 35 min.

Injection volume ranged from 5-10. µl. Quantitative analysis of each sample was calculated by comparing peak area of the samples with that of standards.

Antibacterial activity investigation

The bacteria strains used in present research were from Microbiology Unit at National Research Centre, Egypt. The selected bacteria were *Staphylococcus aureus, Escherichia coli*, *Salmonella typhi*, *Campylobacter jejuni*, and *Listeria monocytogenes*. The selected microorganisms were cultured on agar slopes at 40◦C then sub-cultured into nutrient broth and incubated at 37◦C for 24 hour before sensitivity testing to get more vigorous population. *A. belladonna* mother leaf extract, callus I, callus II extracts (in distilled water) were prepared using doubling dilution method [13] to obtain 12.5mg/ml, 25mg/ml and 50mg/ml (2.5, 5, 10%). The antimicrobial activities of the prepared samples were determined using disk diffusion method (100 µl /disk). About 0.5ml of standardized portion of the microbial culture was aseptically transferred into Petri dishes containing nutrient agar for bacterial isolates and left for about 20 minutes to allow fixation of the microorganisms on the media. Extracts loading disks of various concentrations were introduced into the plates carefully. The plates were incubated at 37◦C and the zones of inhibition were measured in triplicates after 24 hours [14]. The diameter of zones of inhibition was regarded as indication of antimicrobial activity. Zones of inhibition were measured using ruler at right angles across each zone to calculate the average diameter in millimeters. The inhibition zones were compared to four standard broad spectrum antibiotics; erythromycin, amoxicillin (macrolide antibiotic), gentamycin (aminoglycoside antibiotic) and nalidixic acid (synthetic quinolone antibiotic).

RESULTS AND DISCUSSION

The present study showed that calli and regenerated shootlets of *A. belladonna* leaf cultures were effectively produced in MS media contained Kin, BA, and IAA (0.5, 0.5, and 2 mg/l), respectively (culture I), and in MS media supplemented with BA and NAA (0.5 and 1 mg/l) (culture II). For differentiation, shootlets were well developed in Ms media with BA and IAA (0.2 and 2 mg/l). (Figure1).

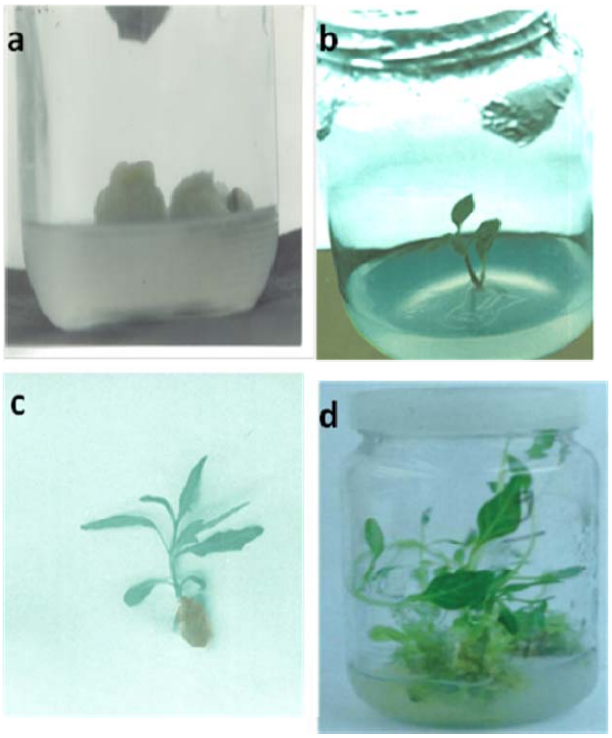

Figure 1. Callus induction and regeneration from *A. belladonna* leaves explants where a: callus induction, b: regenerated shootlets, c: callus and regenerated shoots, d: differentiated plantlets (d; ref. [2].

HPLC analyses clarified the success of *in vitro* cultures of *A. belladonna* callus and differentiated shoots to produce flavonoids and coumarins characteristic for the plant in all the studied extracts. Rutin is the most dominant common flavonoid in the majority of the samples (Table 1).Whereas, quercetin 3-O-rhamnoside (quercitrin) is the unique flavonoid in SMP. Meanwhile, kaempferol and Kaempferol 3-O rhamnoside are the unique flavonoids in LTC and culture I, respectively (Figure 2). Regarding coumarins HPLC analyses illustrated the biosynthesis of bergapten, xanthotoxin and umbelliferone in both intact *A. belladonna* parent leaf plant and different *in vitro* cultures (Figure 3). The results showed that *in vitro* differentiated leaves could biosynthesize simple coumarins and flavonoids only, while *in vitro* regenerated roots and stems were able to biosynthesize complex ones. The data of the bioformed coumarins and flavonoids of calli, regenerated plants and mother leaves are listed in Table 1. The present prosperity of calli and regenerated plants on *in vitro* cultures contained the selected growth regulators (Fig.1), are in harmony with our previous findings [2]. The results proved the ability of callus and differentiation cultures in addition to parent plant to produce flavonoids and coumarins. To our knowledge, no reports have discussed the *in vitro* coumarins and flavonoids production *via A. belladonna* cultures before. Most of the *in vitro* researches concerned with hyoscine, hyoscyamine and atropine alkaloids biosynthesis either in callus, regenerated plants through growth regulators, or in hairy root cultures through *Agrobacterium rhizogenes* [2, 12]. Meanwhile, plant tissue culture was effective tool for coumarins and flavonoids production from other plants [15, 16]. The current results illustrated the variation of coumarins and flavonoids contents among different organs of original plant as well as *in vitro* derived plants. These variations could be influenced by intraspecific diversity that partitioned within the same plant. The difference in the metabolites from culture I and II results from the effect of culture media and growth regulators. It is well documented that culture type and components

play a crucial role in secondary metabolites accumulation. Example of this variation is serpentine production that varied dramatically in cultures of *Catharanthus roseus* according to the utilized basal media and growth regulators [17]. In the same line of the present results is coumarin biosynthesis from *Ammi majus* where xanthotoxin and umbelliferone were not formed in all studied cultures [18].

The current results demonstrated that *A. belladonna* field plant and both calli and regenerated cultures accumulated coumarins and flavonoids phenolics characterized of the [19]. HPLC analyses illustrated that flavonoids and coumarins of field and regenerated plants, are represented by both simple and complex forms in all plant organs except for LTC where the leaves devoid of furanocoumarins and glycosides while umbelliferone and kaempferol are only detected. It's worthy to note the dominating content of furanocoumarins with respect to their biogenetic precursor umbelliferone. This ideally correlated with biosynthetic pathway of furanocoumarin and confirms observations of other authors [15]. In the same line with our findings, it was reported that the secondary metabolites begin to accumulate by the end of exponential growth phase [15]; this can explain the absence of furanocoumarins and flavonoid glycosides in LTC as the leaves are differentiated in later stage and so just the precursors umbelliferone and kaempferol were detected. Table 1 showed that total content of furanocoumarins and umbelliferone ranged from 8.77 to 12.79% mg/g dry wt. in callus culture depending on the culture media and from1.42 to 11.20% mg/g dry wt. in cultured organs, which is equal or higher than that observed in respective plant organs growing under natural conditions in which values ranged from 2.37 to 7.02% mg/g dry wt. The obtained results are not a surprise since *in vitro* organ cultures usually represent higher production of metabolites than collection site which is compatible with the present results; total coumarins in shoots grown under steady artificial illumination (900 lx) was equal or higher than in *R. graveolens* plants developed under natural conditions [20]. Hence, it is proposed that stationary shoot culture could be an available source for getting biologically important furanocoumarins and flavonoids secondary metabolites.

Table 1: Coumarins and flavonoids contents of *A. belladonna* mother plant and *in vitro* cultures

Extracts	Coumarins (% dry wt.)				Flavonoids (% dry wt.)				
	bergapten	xanthotoxin	umbelliferone	total	rutin	quercetin- O-R	kaempferol-O-R	kaempferol	total
Culture I	7.59	0.80	4.40	12.79	-	-	2.89	-	2.89
Culture II	6.18	2.52	0.07	8.77	2.07	-	-	0.25	2.32
LMP	0.99	3.70	2.33	7.02	3.07	0.25	0.04	0.32	3.68
SMP	0.70	0.91	1.24	2.85	-	1.15	-	-	1.15
RMP	0.45	1.70	0.22	2.37	4.63	-	-	-	4.63
LTC	-	-	11.20	11.20	-	-	-	3.10	3.10
STC	0.04	1.27	0.11	1.42	1.41	0.09	-	-	1.50
RTC	0.04	1.37	0.05	1.46	1. 80	0.17	-	0.54	2.51

LMP: leaf mother plant, SMP: stem mother plant, RMP: root mother plant
LTC: leaf tissue culture plant, STC: stem tissue culture plant, RTC: root tissue culture plant.
quercetin- O-R: quercetin -O-rhamnoside, kaempferol-O-R: kaempferol-O- rhamnoside.

Table 2: The effect of callus cultures and intact leaf mother plant of *A. belladonna* on food poisoning pathogenic bacteria

Extracts	Inhibition Zones (mm)				
	S. aureus	*E. coli*	*S. typhi*	*C. jejuni*	*L. monocytogenes*
Culture I					
2.5	13.33± 0.88	11.00±0.57	9.00± 0.58	8.00±0.56	7.67± 0.89
5	15.00±0.56	11.67± 0.86	10.00±1.15	8.67±0.88	8.00± 0.58
10	20.00±0.58	15.48± 0.56	15.00±0.57	10.33±0.34	9.00± 0.87
Culture II					
2.5	19.00±0.55	12.33± 0.87	11.33±1.20	9.00± 0.58	8.00± 0.57
5	21.00±0.57	15.67± 0.89	14.67±0.88	10.00±0.55	10.00± 0.58
10	28.67±0.88	17.00± 0.56	15.67±1.22	12.00±0.59	10.33± 0.88
LMP					
2.5	30±0.8621	12.67± 1.45	12.00±0.55	9.00± 0.58	8.67± 0.33
5	22.30±0.89	16.33± 1.20	15.33±1.76	11.67±0.88	10.67± 0.34
10	30.00±1.15	20.67± 1.23	17.00±0.99	13.33±0.86	11.33± 0.88
Erthromycin15µg	25.40±0.73	-	10.50±1.39	20.60±0.58	6.50±0.88
Gentamycin10µg	24.33±1.76	13.79±1.06	15.50±0.33	16.30±0.87	9.47±1.15
Amoxicillin10µg	29.51±0.64	30.35±0.41	12.00±0.43	12.45±0.32	29.87±0.50
Nalidixic acid 30µg	29.68±0.73	28.95±0.68	-	14.35±0.23	-

S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, S. typhi: Salmonella typhi, C. jejuni: Campylobacter jejuni, L .monocytogenes: Listeria monocytogenes
LMP: Leaf mother plant.

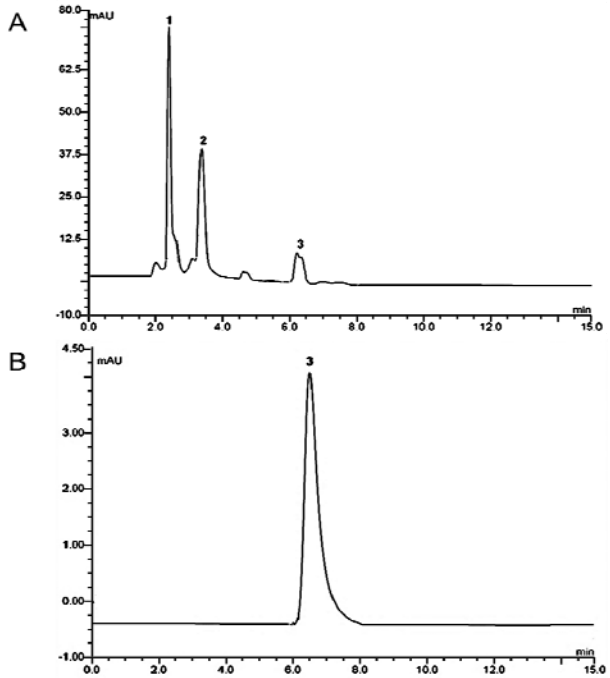

Figure 2. Coumarins of *A. belladonna* where A; root mother plant, B; leaf tissue culture.1: bergapten, 2: xanthotoxin and 3: umbelliferone

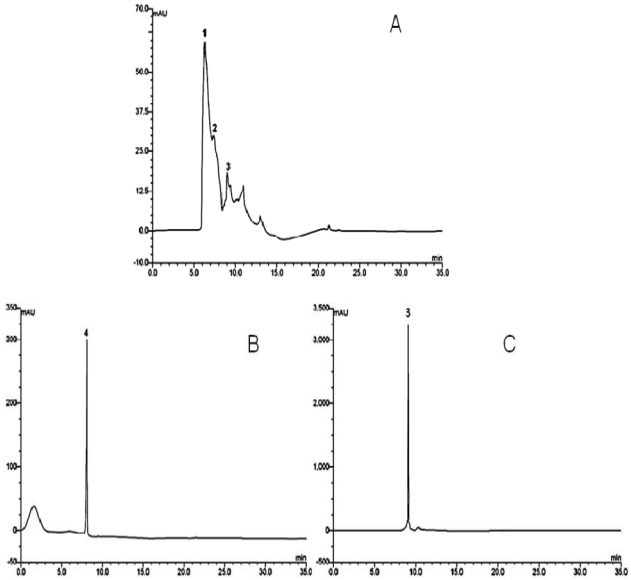

Figure 3. Flavonoids of *A. belladonna* where A; root tissue culture, B; culture I and C; leaf tissue culture. 1: rutin, 2: quercetin rhamnoside, 3: kaempferol and 4: kaempferol rhamnoside.

With respect to antibacterial activity, the present outcomes expound the promising antimicrobial activity of callus cultures (I and II) and leaf mother plant (LMP) against the majority of selected food poisoning bacteria (Table 2). The three chosen concentrations (2.5, 5 and 10%) exhibited antimicrobial activity against all the tested organisms represented by inhibition of growth that was parallel to the concentration of the tested samples. The most effective concentration was 10% extract regarding leaf mother plant, culture I and culture II. The extract from mother plant (LMP) demonstrated higher antibacterial activity than that of both callus cultures I and II. The inhibition zones diameters were in decreasing order from *S.aureus*> *E.coli*>*S. typhimurium*>Campylobacte>*Listeria*. In addition, current results illustrated that the antimicrobial activity of the samples were directly correlated to xanthotoxin coumarin and rutin flavonoid concentrations. The present results are matched with previous studies, which reported *A. belladonna* ethanolic extracts to exhibit higher antimicrobial activity against *S. aureus* than *E. coli* [8]. However, in contrast to our results, the authors reported that *A. belladonna* extracts showed no activity against *S. typhi*, while belladonna examined extracts in the current study, exhibited promising antibacterial activity against *S. typhi* that was higher than all compared antibiotics at the highest tested dose (10%) and approximately the same activities as the antibiotics at the median dose (5%) for mother plant and culture II. According to Kirby-Bauer Disc sensitivity for inhibition zones [21, 22]; the results indicated that *S. typhi* was resistant to erythromycin, amoxicillin, nalidixic acid (≤13mm for all) and sensitive to gentamycin. The current data also revealed that all the tested plant materials at their highest dose were as sensitive as gentamycin where zones of inhibitions were in the domain of gentamycin susceptibility (≥ 15 mm) (Table 2). Besides, the data as well, showed that *S. aureus* was highly sensitive to all antibiotics used for comparison according to Kirby-Bauer calibration. It is interesting that both mother plant and culture II extracts exhibited almost the same activities as the antibiotics at 10% concentrations, while culture I was less active but still within the sensitivity range of the antibiotics according to susceptibility test [21,22] (Table 2). Following the same sensitivity measurements, *C. jejuni* was sensitive to erythromycin (and gentamycin (≥ 18, ≥ 15mm respectively) and showed intermediate activity towards nalidixic (14-18mm) acid but was resistant to amoxicillin (≤13mm). Only mother-tested extract showed as intermediate activity as gentamycin (13-14mm) (Table 2). Regarding *L. monocytogenes*, it was found to be resistant to erythromycin, nalidixic acid and gentamycin as well but was highly sensitive to amoxicillin where sensitivity inhibition zone for amoxicillin is (≥18mm). The results also indicated that the inhibition zones exhibited by the plant materials were less than all compared antibiotics (Table 2). Meanwhile, *E. coli* was the most resistant to erythromycin and showed intermediate sensitivity to gentamycin and high sensitivity to amoxicillin and naldixic acid (≥18, 19mm respectively). Although the plant materials at 10% concentrations performed less inhibition zones than the antibiotics, yet mother plant was in the range of reported sensitivity of all antibiotics. Culture I and II were in the sensitivity range as gentamycin (≥15mm) and as intermediated sensitivity of erythromycin (13-17mm) and nalidixic acid (14-18mm) [21, 22] (Table 2). The antibacterial activity herein is attributed to the coumarins and flavonoids disclosed in the extracts. The antimicrobial activity of the three detected coumarins and rutin were previously documented [23, 24]. The results reflect a correlation between the concentrations of the compounds and the potency of the extracts; it seems there is a direct correlation between the growth inhibition effect and the concentrations of xanthotoxin and rutin in the extracts. Xanthotoxin was documented to have antibacterial activities against both gram-positive; *S. aureus* and gram-negative strains; *E. coli* and *S. typhimurium* [25]. A reported study evaluated the antimicrobial activity of some flavonoids including rutin against *S. aureus* and. *E.coli* [24]. In Another study, afzelin (Kaempferol rhamnoside), showed potent antimicrobial effect versus *S. aureus* and *S. typhi* [26]. Despite the reported lower antibacterial effect of flavonoids than the antibiotics, recent studies adduced the additive action of quercetin and rutin when used in combination with common antibiotics where the antibacterial activities of both flavonoids and antibiotics were markedly enhanced [27].

CONCLUSION

Access to adequate safe antimicrobial agents is basic requirement for human health as food poisoning pathogens lead to serious health complications. According to WHO, Food safety fact sheet N°399 in 2015, about 600 millions fall ill through food contamination with increasing number of death annually especially between children in poor countries. Among the most reported common food borne bacteria that affect millions of people annually, are Salmonella, Campylobacter, Enterohemorrhagic *E. coli* and Listeria sometimes with dangerous health consequences and withering outcomes. The hazards of unsafe food are serious, and hard to quantify. Therefore, the repression of various microbial pathogenic isolates in the current research by *A. belladonna* is of particular attention. The present outcomes could be extrapolated to interpret its efficiency in the handling of infections caused by these food poisoning bacteria, which involved in critical ailments. Besides, to overcome the problem of emerge of bacterial resistance to common antibiotics in addition to their side effects. Furthermore, the study pointed to tissue culture technique as helpful approach in stabilizing belladonna phenolics supply that could be developed to natural antibacterial medication.

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